CONSIDERED: /RAZ/

07/27/2009

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the patent application of: Guzman and Mühlradt

Serial No. 10/509,917 Group Art Unit 1645 Confirmation No: 4470

Filed 10/04/2004 Examiner: Zeman

For: *"USE OF A LIPOPEPTIDE OR LIPOPROTEIN AS AN ADJUVANT IN THERAPEUTIC OR PROPHYLACTIC VACCINATIONS"*

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132
OF DR. Peter F. Mühlradt

Sir:

1. I have attached my *Curriculum vitae* to this declaration. In short, I received the degree Doctorate of Chemistry at the University of Switzerland in Basel, Switzerland in 1964. I was employed as a post-doc in the Department of Biochemistry at the University of California at Berkeley, CA from 1964-1966, where I worked on vitamin B6 analogues. For 1966 -1975, I was employed at the Max Planck Institute for Immunobiology in Freiburg, Germany, working on the biosynthesis of bacterial cell walls, specifically lipopolysaccharide endotoxins. In 1975, I was called to lead the immunology research group at the Gesellschaft for Biotechnological Research Ltd. in Braunschweig, Germany. Until my retirement in 2000, I also taught immunology at the University of Braunschweig and worked on several aspects of immunology such as interleukins, carbohydrate differentiation antigens and a substance with endotoxin-like properties from mycoplasmas. In the course of my work, I elucidated the structure of this compound which was named MALP-2 (macrophage activating lipopeptide of 2 kDa). My research then focused on the biological properties of MALP-2. After my retirement, I founded a small private research group "Wound Healing/MALP Research" in Braunschweig. As evidenced by my *Curriculum vitae* and the summary above, I qualify as an "expert" in the fields of medicinal chemistry and immunology, and I am able to provide evidence on matters pertaining to these fields and on matters pertaining particularly to immunobiology and lipopeptides. I am also qualified to provide evidence on the level of skill of one or ordinary skill in the art.

2. In my expert opinion, one of skill in the art is a person with a doctoral degree, 5-10 years of research experience in immunology or related fields, and an author of 10 or more peer-reviewed articles. He or she would be familiar with adjuvants as well as concepts related to macrophage stimulation.

3. I am an inventor of the above-identified application. I have reviewed the subject patent application, including the claims, and the Examiner's remarks as contained in the Office Action mailed on December 12, 2008. I have also reviewed the cited references, and note that the joint inventor on the present application is the author or inventor of those references (Muhlradt, *J. Experimental Medicine*, 1997, Vol. 185, No. 11, pages 1951-1958; U.S. Patent 6,573,242 to Muhlradt and its priority documents).

4. Regarding the stated position that it would be obvious for a skilled artisan to use the S-(2, 3-dihydroxypropyl)-cysteine peptide (MALP-2) disclosed in Muehlradt (US patent 6,573,242) as a mucosal adjuvant, it is my expert opinion that a macrophage stimulator such as MALP-2 is not *a priori* an adjuvant, and in particular, is not necessarily a mucosal adjuvant. While macrophage stimulation, resulting primarily in an inflammation, may be a "*conditio sine qua non*" for raising an immune response, <u>it is, not, in and of itself, sufficient to raise an immune response</u>. Although macrophages, and also fibroblasts and B lymphocytes, are capable of presenting antigens, the pivotal cell involved in antigen presentation is the dendritic cell. As evidence thereof, page 647, 4th paragraph of Immunobiology, 6th Ed. Janeway, Travers, Walport, Shlomchik, 2005, states that: "It is thought that most, if not all adjuvants act on antigen-presenting cells, especially on dendritic cells, and reflect the importance of these cells in initiating immune responses".

5. The priority date of the cited Muehlradt patent is December 17, 1998 (priority to German patent application 196 52.586). The publication date of the Muhlradt article in *Journal of Experimental Medicine* is in 1997. Both references were published several years before the effectiveness of MALP-2 as a mucosal adjuvant and its ability to act on dentritic cells were known. The effectiveness of MALP-2 as a mucosal adjuvant was first described in 2002 in "The Mycoplasma-derived lipopeptide MALP-2 is a potent mucosal adjuvant". Rharbaoui F, Drabner

B, Borsutzky S, Winckler U, Morr M, Ensoli B, Mühlradt PF, Guzmán CA. *Eur J Immunol.* 2002 Oct;32(10):2857-65". The first paper showing that MALP-2 acts on dendritic cells appeared even later, in 2003: "Synthetic mycoplasma-derived lipopeptide MALP-2 induces maturation and function of dendritic cells". Weigt H, Mühlradt PF, Emmendörffer A, Krug N, Braun A. *Immunobiology.* 2003;207(3):223-33". Thus, the effectiveness of MALP-2 as a mucosal adjuvant and/or its ability to act on dendritic cells could by no means have been obvious to one of skill in the art with a knowledge of the prior Muehlradt patent, at the time of filing of the present application. Further, he present application claims prior to filings which pre-date the two articles noted in this section of my declaration.

6. In addition, it is my opinion that one of ordinary skill in the art would know that the effectiveness of a known adjuvant in a new experimental setting cannot be foreseen. This is documented in two representative publications (copies enclosed) which can be taken as evidence in support of this statement. The first is "Safety and immunogenicity of a recombinant *Plasmodium falciparum* AMA1 malaria vaccine adjuvanted with Alhydrogel, Montanide ISA 720 or AS02. Roestenberg M, Remarque E, de Jonge E, Hermsen R, Blythman H, Leroy O, Imoukhuede E, Jepsen S, Ofori-Anyinam O, Faber B, Kocken CH, Arnold M, Walraven V, Teelen K, Roeffen W, de Mast Q, Ballou WR, Cohen J, Dubois MC, Ascarateil S, van der Ven A, Thomas A, Sauerwein R. PLoS ONE. 2008;3(12):e3960. Epub 2008 Dec 18".
The authors of this article state in their conclusions that "All formulations showed distinct reactogenicity profiles". In other words, each formulation tested showed a different reaction profile and effectiveness with respect to immunogenicity. A second article is "Expert Rev Vaccines. 2007 Oct;6(5):723-39. GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. Garçon N, Chomez P, Van Mechelen M.GlaxoSmithKline Biologicals, Research & Development, 1330 Rixensart, Belgium". The authors of this article state in the abstract that:"Adjuvant systems are formulations of classical adjuvants mixed with immunomodulators, specifically adapted to the antigen and the target cell population." In other words, the effects of adjuvant formulations differ and depend at least in part on the antigen and/or target cell population. The existence of ongoing adjuvant research programs at pharmaceutical companies such as GlaxoSmithKline is evidence of an unmet need in the field, and provides further evidence that the experimental outcomes of current adjuvant research are

not obvious or readily predictable by those of skill in the art.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application and any patent issuing thereon.

Date May 10, 2009 Signed P. Mühlradt

Peter F. Mühlradt

CURRICULUM VITAE

Personal:

Name: Peter F. Mühlradt
born: 11th of Jan. 1937 in Hamburg, Germany
marital status: married with Toni Mühlradt since 1972

Education:

1956: Senior matriculation (Abitur)
56-57: Laboratory Assistant in pharmaceutical industry in Montreal, Canada
57-62: University of Hannover/Germany and Basle, Switzerland. Majors: Organic and Inorganic Chemistry, Minors: Physics, Pharmacology, Physical Chemistry
62-64: PhD thesis with T. Reichstein on Cardiac Glycosides in Seeds of "Antiaris toxicaria Lesch"

Professional Experience, Positions held:

64-66: Postdoc at Univ. of California, Berkeley with E. E. Snell (Fulbright Travel Stipend). Synthetic work on vitamin B_6 analogues.
66-75: Postdoc at the Max Planck Institute for Immunobiology at Freiburg/Germany. Research work on biosynthesis and translocation of lipopolysaccharide, topography of the outer membrane of Gram-negative bacteria

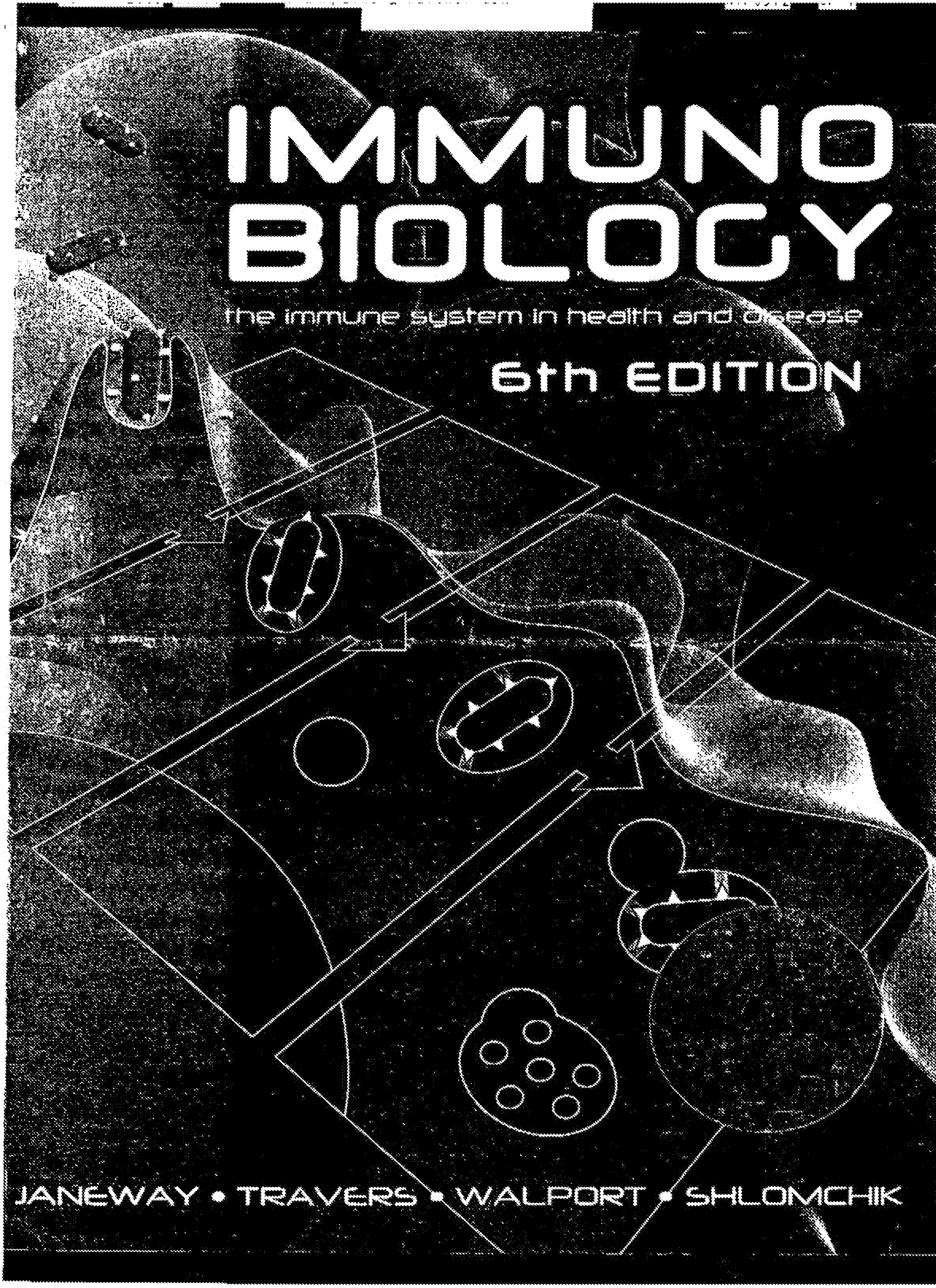
since 75: Head of the Immunology Research Group at the Gesellschaft für Biotechnologische Forschung m.b.H GBF, Braunschweig/Germany. Research on glycoconjugates of lymphoid cells; pilot plant production of lymphokines; macrophage differentiation antigens, macrophage activation by mycoplasma products. Structure of macrophage-activating mycoplasmal lipo-peptides and -proteins. Work on toll-like receptor agonists.

since 81: Professor of Biochemistry at the University of Braunschweig. Lecturing on bacterial membranes, introduction to immunology. About 100 publications in refereed journals. Review articles in books.

2002: retirement from GBF (now HZI). Self employed.

Founder of Wound Healing Research Group at the BioTec Gründerzentrum Braunschweig. Research on wound healing in animal models. Consulting research with MALP-2. Phase 1 clinical studies in a wound healing (T. Werfel, Med.School Hannover) and a pancreas cancer project (A. Märten, Med.School Heidelberg) with MALP-2.

2008 Founding of MBiotec Ltd.


IMMUNO
BIOLOGY
the immune system in health and disease
6th EDITION
JANEWAY • TRAVERS • WALPORT • SHLOMCHIK

Capsular polysaccharides can be harvested from bacterial growth medium and, because they are T-cell independent antigens, they can be used on their own as vaccines. However, young children under the age of 2 years cannot make good T-cell independent antibody responses and cannot be vaccinated effectively with polysaccharide vaccines. An efficient way of overcoming this problem (see Fig. 9.5) is to conjugate bacterial polysaccharides chemically to protein carriers, which provide peptides that can be recognized by antigen-specific T cells, thus converting a T-cell independent response into a T-cell dependent anti-polysaccharide antibody response. By using this approach, various conjugate vaccines have been developed against *Haemophilus influenzae* type b, an important cause of serious childhood chest infections and meningitis, and against *Neisseria meningitidis* serogroup C, and these are now widely applied.

14-21 The use of adjuvants is another important approach to enhancing the immunogenicity of vaccines.

Purified antigens are not usually strongly immunogenic on their own, and most acellular vaccines require the addition of adjuvants, which are defined as substances that enhance the immunogenicity of antigens (see Appendix I, Section A-4). For example, tetanus toxoid is not immunogenic in the absence of adjuvants, and tetanus toxoid vaccines often contain aluminum salts, which bind polyvalently to the toxoid by ionic interactions and selectively stimulate antibody responses. Pertussis toxin, produced by *B. pertussis*, has adjuvant properties in its own right and, when given mixed as a toxoid with tetanus and diphtheria toxoids, not only vaccinates against whooping cough but also acts as an adjuvant for the other two toxoids. This mixture makes up the DTP triple vaccine given to infants in the first year of life.

Many important adjuvants are sterile constituents of bacteria, particularly of their cell walls. For example, Freund's complete adjuvant, widely used in experimental animals to augment antibody responses, is an oil and water emulsion containing killed mycobacteria. A complex glycolipid, muramyl dipeptide, which can be extracted from mycobacterial cell walls or synthesized, contains much of the adjuvant activity of whole killed mycobacteria. Other bacterial adjuvants include killed *B. pertussis*, bacterial polysaccharides, bacterial heat-shock proteins, and bacterial DNA. Many of these adjuvants cause quite marked inflammation and are not suitable for use in vaccines for humans.

It is thought that most, if not all, adjuvants act on antigen-presenting cells, especially on dendritic cells, and reflect the importance of these cells in initiating immune responses. As we saw in Section 8-6, dendritic cells are widely distributed throughout the body, acting as sentinels to detect potential pathogens at their portals of entry. These tissue dendritic cells take up antigens from their environment by phagocytosis and macropinocytosis, and they are tuned to respond to the presence of infection by migrating into lymphoid tissue and presenting these antigens to T cells. They seem to detect the presence of pathogens in two main ways. The first of these is direct, and follows the ligation and activation of receptors for invading microorganisms. These include receptors of the complement system, Toll-like receptors (TLRs), and other pattern recognition receptors of the innate immune system (see Chapter 2).

The discovery that the effects of many adjuvants are mediated by the activation of TLRs on dendritic cells opens the door to the rational development of novel adjuvants for vaccine therapy. Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria. It has adjuvant effects but

PLoS one

Safety and Immunogenicity of a Recombinant *Plasmodium falciparum* AMA1 Malaria Vaccine Adjuvanted with Alhydrogel^{TM}, Montanide ISA 720 or AS02

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Abstract

Background: *Plasmodium falciparum* Apical Membrane Antigen 1 (*Pf*AMA1) is a candidate vaccine antigen expressed by merozoites and sporozoites. It plays a key role in red blood cell and hepatocyte invasion that can be blocked by antibodies.

Methodology/Principal Findings: We assessed the safety and immunogenicity of recombinant *Pf*AMA1 in a dose-escalating, phase Ia trial. *Pf*AMA1 FVO strain, produced in *Pichia pastoris*, was reconstituted at 10 µg and 50 µg doses with three different adjuvants, *Alhydrogel*^{TM}, Montanide ISA720 and AS02 Adjuvant System. Six randomised groups of healthy male volunteers, 8–10 volunteers each, were scheduled to receive three immunisations at 4-week intervals. Safety and immunogenicity data were collected over one year. Transient pain was the predominant injection site reaction (80–100%). Induration occurred in the Montanide 50 µg group, resulting in a sterile abscess in two volunteers. Systemic adverse events occurred mainly in the AS02 groups lasting for 1–2 days. Erythema was observed in 22% of Montanide and 59% of AS02 group volunteers. After the second dose, six volunteers in the AS02 group and one in the Montanide group who reported grade 3 erythema (>50 mm) were withdrawn as they met the stopping criteria. All adverse events resolved. There were no vaccine-related serious adverse events. Humoral responses were highest in the AS02 groups. Antibodies showed activity in an *in vitro* growth inhibition assay up to 80%. Upon stimulation with the vaccine, peripheral mononuclear cells from all groups proliferated and secreted IFNγ and IL-5 cytokines.

Conclusions/Significance: All formulations showed distinct reactogenicity profiles. All formulations with *Pf*AMA1 were immunogenic and induced functional antibodies.

Trial Registration: Clinicaltrials.gov NCT00730782

Citation: Roestenberg M, Remarque E, de Jonge E, Hermsen R, Blythman H, et al. (2008) Safety and Immunogenicity of a Recombinant *Plasmodium falciparum* AMA1 Malaria Vaccine Adjuvanted with Alhydrogel^{TM}, Montanide ISA 720 or AS02. PLoS ONE 3(12): e3960. doi:10.1371/journal.pone.0003960

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Competing Interests: AS02 is the proprietary Adjuvant System from GlaxoSmithKline Biologicals, which might pose a conflict of interest to associated authors. Other authors do not have a commercial or other association that might pose a conflict of interest.

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Introduction

In sub-Saharan Africa the burden of death and disease from *Plasmodium falciparum* malaria is particularly severe. To date, there are no approved vaccines to help reduce this burden, although a number of candidate vaccines have been put forward. The majority of the candidates target the pre-erythrocytic circumsporozoite protein (CSP) and the merozoite proteins Merozoite Surface Protein 1 (MSP1) and Apical Membrane Antigen 1 (AMA1)[1]. The RTS,S candidate vaccine has shown efficacy in infants and children [2,3] and a phase III clinical trial is planned. The MSP1 and AMA1 candidate vaccines are in early stage clinical development and efficacy trials will provide information to determine whether these antigens are suitable targets, and whether they can be deployed singly or as components of a multivalent malaria vaccine.

Following an infected mosquito bite, *P. falciparum* sporozoites migrate to hepatocytes, each developing over a period of a week to release several thousand merozoites. These initiate cyclical asexual blood stage development, producing merozoites that invade erythrocytes. AMA1 is an integral membrane protein of merozoites and sporozoites and has a central role in parasite invasion of erythrocytes and potentially hepatocytes that can be inhibited by anti-AMA1 antibody [4–6]. In merozoites, AMA1 is synthesised as an 83 kDa molecule originally localised to the microneme. Around the time of merozoite release and the subsequent rapid erythrocyte invasion, the protein is N-terminally cleaved to a 66 kDa form. This translocates to the merozoite surface and undergoes secondary proteolytic processing, shedding soluble fragments (44 or 48 kDa) [7].

Immunisation with AMA1 can provide protection against infection in experimental animal models, and can induce antibodies that show functionality in *in vitro* growth inhibition assays (GIA). However, AMA1 is polymorphic and immune responses have varying degrees of strain specificity and growth inhibition [8].

Previous Phase I trials have shown that growth inhibitory antibodies can be induced by immunisation with *Pf*AMA1 [9,10], but immunogenicity varied depending on the vaccine formulation. In particular, the choice of adjuvant has a major effect on the safety, stability, immunogenicity and, presumably, eventual efficacy of a vaccine [11]. Adjuvants can be tools that channel the immune reponse to generate high levels of the desired type of long-lived immunity. *Alhydrogel*™, an aluminum salt, is the most widely used adjuvant in licensed human vaccines and is therefore used as a standard to compare other adjuvants. Unfortunately, in combination with malaria antigens, it has generally induced poor reponses [12–15]. Montanide ISA 720, a squalene based water-in-oil adjuvant formulation has shown promising results in previous malaria vaccine trials [16–19], possibly due to the slow-release capacity of the inert water-in-oil emulsion and immune stimulating effects of its components [20]. AS02, a proprietary Adjuvant System from GlaxoSmithKline Biologicals based on an oil-in-water formulation, contains 50 µg each of the immunostimulants monophosphoryl lipid A (MPL) and Quillaja saponaria 21 (QS21) [21]. It has been used to adjuvant the RTS,S malaria candidate vaccine that targets CSP. To date, this candidate is the only malaria vaccine that has induced protection in adults, children and infants in natural field trials [22–25]. When combined with *Alhydrogel*™, RTS,S did not convey protection in a combined phase I/IIa trial [26]. AS02 is capable of eliciting high antibody titers along with strong cell-mediated immunity [27], both of which are believed to contribute to the efficacy of the RTS,S candidate vaccine [28].

Because of their central role in vaccine formulation, the development of adjuvants and delivery systems have become increasingly important. This study aims at comparing the safety and immunogenicity of *Pf*AMA1 in two dosages formulated with three different adjuvants in a phase Ia trial.

Materials and Methods

Vaccine preparation

Clinical grade *Pf*AMA1-FVO [25-545] was developed[29] and produced[30] as previously reported. In brief, FVO strain *Pf*AMA1 was codon adapted to expression in the methylotrophic yeast *Pichia pastoris*. Glycosylation sites were conservatively mutated, and the ectodomain comprising amino acids 25–545 was expressed. *Pf*AMA1-FVO[25-545] preparation was manufactured and lyophilised according to current good manufacturing practice in multidose vials containing either 120 µg (44 µg EDTA, 180 µg sucrose and 120 µg $NaHCO_3$, lot B) or 62.5 µg (23 µg EDTA, 25 mg saccharose, 226 µg K_2HPO_4 and 187 µg NaH_2PO_4, lot C) of AMA1 that were stored between −18°C and −30°C and between +2 and +8°C respectively. Quality control and stability data are described by Faber et al. [31]. Reconstitution and mixing of vaccine with adjuvant was performed under sterile conditions under responsibility of the hospital pharmacist.

*Pf*AMA1 vaccine at 50 µg (high dose) and 10 µg (low dose) *Pf*AMA1 per injection (0.5 ml) was formulated with three different adjuvants and, after preparation, was kept at a constant temperature of +4°C for a maximum of six hours until injection. For the *Alhydrogel*™ formulation, 1.2 ml aluminum hydroxide suspension at 2 mg/ml (Statens Serum Institut (SSI), Copenhagen, Denmark) was added to the 120 µg *Pf*AMA1 vial (lot B) to obtain a high dose (50 µg in 0.5 ml) and 6 ml was added to obtain a low dose (10 µg in 0.5 ml) formulation. The resulting amount of aluminum in each vaccine was 0.5 mg. Stability studies confirmed adsorbtion of 99.9% of the antigen to the aluminum. Montanide formulations were prepared by dissolving the contents of the 120 µg *Pf*AMA1 vial (lot B) in sterile phosphate buffered saline (145 mM NaCl, 5 mM Phosphate, pH 7.4), 0.32 ml for the high dose and 1.6 ml for the low dose formulation. Montanide ISA 720 (SEPPIC, Paris, France) was subsequently added, 0.88 ml for the high dose to obtain 1.2 ml of formulation (50 µg *Pf*AMA1 in 0.5 ml) and 4.4 ml for the low dose to obtain 6 ml of formulation of which five 10 µg *Pf*AMA1 in 0.5 ml doses could be prepared. The suspension was prepared by manually pushing through a 22 gauge syringe coupling piece (3038068 Omnilabo International, Breda, The Netherlands) at +20C° for twenty up and down strokes.

The suspension was confirmed to be homogeneous and reached a median droplet size of approximately 1.5 µm (SD 0.17 µm) by particle size measurements with the Malvern Mastersizer S by SEPPIC.

For the AS02 formulation, the contents of one vial of lyophilized *Pf*AMA1 containing 62.5 µg of antigen (lot C) was mixed by gentle shaking with AS02 (approximately 0.6 ml) [32]. A 0.5 ml dose contained approximately 50 µg AMA-1 in 500 µl AS02 (high dose). For low dose preparations (10 µg) five times more AS02 adjuvant was added to the 62.5 µg vial of AMA1, from which five 0.5 ml low vaccine doses could be obtained.

Study design

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1 with Amendments S2, S3, S4, S5, S6 and S7. The study was designed as a dose-escalating phase Ia trial to assess the safety and immunogenicity of two dosages of *Pf*AMA1 with three different adjuvants. Volunteers were thus randomised into six different groups, each of which was aimed to constitute of a limited number of 10 volunteers for safety reasons. Randomisation was performed by an external statistician in six blocks through a computer program. Block randomization were used to ensure equal distribution of adjuvants among the immunisation groups. There was no stratification for sex and/or age. The randomization list was provided to the pharmacy departments. The clinical investigators allocated the next available number on entry into the trial. The code was revealed to the researchers once recruitment, data collection, and laboratory analyses were complete. The immunisations were thus performed blind, so neither volunteers, nor investigator or laboratory personnel were aware of the adjuvant allocation. Because of the dose-escalating design, the trial could not be blinded for dose.

For logistical reasons, the AS02 adjuvanted groups were immunised nine months after the *Alhydrogel*™ and Montanide groups, breaking the blind for this trial arm. A subsequent bias cannot formally be excluded but seems unlikely, since all trial procedures were identical. All immunisations were performed intramuscularly in the deltoid region of alternate arms at 0, 4 and 8 weeks.

Participants

We aimed to recruit 60 healthy, malaria naïve male volunteers, aged between 18 to 45 years through advertisements at the Radboud University Nijmegen Medical Centre. Potential volunteers provided a medical history and a physical examination was conducted with routine laboratory tests consisting of full blood count, serum biochemistries and serologic assays for human immunodeficiency virus, hepatitis C and B virus. Volunteers were excluded from participation if they had any symptoms, signs or laboratory values suggestive of systemic illness, including renal, hepatic, cardiovascular, pulmonary, skin, immunodeficiency, psychiatric and other conditions, which could interfere with the interpretation of the study results or compromise the health of the volunteers, or received chronic medication, had a history of drug or alcohol abuse interfering with social function one year prior to enrolment, or a known hypersensitivity to any of the vaccine components. Additional reasons for exclusion were a history of malaria or residence in malaria endemic areas within the past six months, previous participation in a malaria vaccine trial or receiving vaccines other than the study vaccines. Furthermore, volunteers were not enrolled in any other clinical trial, and agreed to remain available to be closely monitored. All volunteers provided written informed consent. The study was approved by the Institutional Review Board (CMO Regio Arnhem-Nijmegen, 2005/015). The study was conducted in accordance with the Declaration of Helsinki principles for the conduct of clinical trials and the International Committee of Harmonization Good Clinical Practice Guidelines [33] and registered at www.clinicaltrials.gov (NCT00730782).

Assessment of safety

Volunteers were observed for 30 minutes and evaluated on days 1, 3, 7 and 14 after every immunisation. At each visit, local and systemic reactogenicity was assessed by a physician and findings recorded and scored as follows: grade 1, mild reaction (easily tolerated), grade 2, moderate reaction (interferes with normal activity), or grade 3, severe reaction (prevents normal activity). Redness, swelling and induration (according to Brighton collaboration definitions, www.brightoncollaboration.org) were measured with a ruler, and categorised according to the longest diameter as grade 1: ≤20 mm, grade 2: >20 and ≤50 mm, grade 3: >50 mm. Temperature was measured with an oral thermometer; fever intensity was defined as grade 1 (37.5°C to 38°C), grade 2 (>38°C to 39°C) or grade 3 (>39°C). The following adverse events were solicited and recorded routinely during the 14 days after immunisation: injection site pain, redness and swelling, systemic fatigue, fever, headache, malaise, myalgia, joint pain, gastrointestinal symptoms and contralateral local reactions.

Blood samples

Safety was also determined by serial laboratory evaluations of clinical chemistry and haematology on blood samples collected 7 and 28 days after immunisation. For evaluation of immunogenicity, blood was collected in Vacutainer CPT tubes (Becton and Dickinson) and processed within two hours after collection on immunisation days, one month after each immunisation and on Days 140 and 365. Plasma was collected after centrifugation (2000 g 15′) aliquoted and stored at −20°C for antibody analysis (ELISA, Immuno Fluorescence Assay (IFA) and Growth Inhibition Assay (GIA)). Peripheral blood mononuclear cells (PBMC) were collected, washed in PBS (800 g, 10 min) and immediately used for assays (lymphocyte stimulation assay and ELISPOT).

Measurement of anti-AMA1 antibodies by ELISA and IFA

Antibody to *Pf*AMA1 was measured using a standardized ELISA protocol. All procedures used Phosphate buffered saline (PBS) and for washing steps 0.05% Tween 20 (Sigma-Aldrich). Briefly, wells in 96-well polystyrene plates (NUNC Maxisorp, Sanbio), were coated overnight (100 µl, 0.5 µg/ml *Pf*AMA1, 4°C), washed (3×), blocked (60 min, 3% BSA (Sigma-Aldrich)) and washed (3×) before addition of 100 µL from duplicate dilution series (diluted in PBS-Tween BSA, one hour, +37°C). After washing (3×) goat anti-human IgG alkaline phosphate (Perbio Science) diluted 1:1250 in 0.5% BSA, 0.05% Tween was added, (one hour, +37°C). Plates were washed and 100 µl of 1 mg/ml para-nitro-phenyl-phosphatase (Fluka, Sigma-Aldrich) substrate was added (30 minutes, room temperature). A human plasma pool from a malaria endemic area was used as reference positive control, whereas a plasma pool from eight healthy malaria-naive Dutch volunteers was used as a negative control. Optical density was measured at 405 nm. Variation between duplicates was set to a maximum of 15%. Measurements with a greater variation were repeated. The standard curve of human plasma pool from a malaria endemic area, defined to contain 400 Arbitrary Units, was fitted to a four-parameter hyperbolic function, using the ADAMSEL program (E. Remarque, unpublished work). Using this standard curve, optical density from samples were converted to Arbitrary Units (AU). Test samples that did not fall within the linear part of the optical density range of the standard were tested at alternate dilutions.

IFA was performed on cultured *P. falciparum* parasitized red blood cells. Ten well black slides (30-966-A black, Nutacon, The Netherlands) were coated with a washed parasite suspension of 3×10^6 parasites/ml, air dried and kept at −80°C until used. FCR3 parasites, expressing an AMA1 protein with one amino-acid difference from the FVO parasites, and NF54 strain parasites, with 26 amino-acid difference in AMA1 protein were used to prepare slides.

Based on antibody titers by ELISA on day 84, a representative sample of fifteen sera was selected for IFA, containing at least two samples from each adjuvant group and at least three samples with low, intermediate or high ELISA titers. Before use slides were brought to room temperature in an evacuated exicator. Plasma was diluted in PBS (1:40, 1:80, 1:160, 1:320, 1:640) and a final volume of 20 µL was added to the wells and incubated for 0.5 hour, at room temperature. As for the ELISA protocol, the malaria-naïve blood bank donor plasma pool was used as a negative control and human malaria endemic plasma was used as a positive control. After washing (2× in PBS) and air drying, slide samples were incubated with rabbit anti-human Immunoglobin FITC (F0200, DAKO, Denmark) in 0.05% w.v Evans Blue (3169, Merck), PBS for 30 minutes at room temperature. Slides were washed twice and incubated for 15 minutes with DAPI (4′-6-Diamindino-2-phenylindole, 24653, Merck, Darmstadt, Germany), 5 µg/ml in PBS. After washing (2× in PBS) slides were mounted with Vectashield Mounting Medium (H-1000, Brunschwig, Amsterdam), covered with a deck-slide and read immediately by two independent blinded examiners. Examiners identified the highest dilution still showing a staining pattern above the background of pre-immunisation samples. Differences between examiners were never greater than one dilution and the mean of both dilutions was taken.

ELISPOT for IFNγ and IL-5

ELISPOT was performed according to manufacturer's instructions (Becton and Dickinson Elispot Set Human IFNγ or IL-5). In summary, plates provided in the set were coated with either IFNγ or IL-5 capture antibody (5 μg/ml, overnight, 4°C). After blocking with complete medium solution (RPMI 1640 (Invitrogen) containing 10% Fetal Bovine Serum (FBS Invitrogen, Breda, The Netherlands), 1% Glutamax (Invitrogen), 1% Penicillin-Streptomycin (GIBCO-BRL, Invitrogen), 1% MEM) 100 μl of 10^5 PBMC suspension and 100 μl of *Pf*AMA1 containing either 60 μg, 12 μg or 2.4 μg was added per well. Positive controls were stimulated with Tetanus Toxoid 10 μg/ml (RIVM, Bilthoven, The Netherlands) and phytohaemagglutinin 5 μg/ml (PHA-L Sigma-Aldrich) end concentration. Negative controls were incubated with complete medium solution (mean SFC/10^5 cells 31 ± 17 for IFNγ and 9 ± 7 for IL-5). After incubation (40 hours, 37°C in humidified 5% CO_2), biotinylated anti human IFNγ and IL-5 (0.25 μg/ml and 2 μg/ml, respectively), containing 10% FBS was added (two hours at room temperature). Streptavidin-HRP was used as an enzyme conjugate. Detection was performed with the Becton and Dickinson AEC Substrate Reagent Set, according to manufacturer's instruction. Spot-forming cell numbers were counted by ELISPOT reader (4 Microtiter Plate Reader, AELVIS, Sanquin, Amsterdam) and analysed by the ELISPOT Analysis Software Version 4.0 (Sanquin, Amsterdam). All measurements were performed in triplo. Variation between triplicates was set to a maximum of 20%.

Lymphocyte Stimulation Assay

Lymphocyte stimulation assays were performed as described previously [34]. Peripheral blood mononuclear cell suspension (PBMC) was diluted to 1×10^6 PBMC per ml in Dulbecco's MEM (DMEM) with Glutamax-I, 2 mM pyruvate and high Glucose (GIBCO BRL, Invitrogen) supplemented with 10 mM HEPES buffer (GIBCO BRL, Invitrogen), 100 IU/mL Penicillin-Streptomycin (GIBCO BRL, Invitrogen), 100 μM non-essential aminoacids (GIBCO BRL, Invitrogen) and 2.5% human AB serum (AB) (Bodinco BV, Alkmaar, The Netherlands). 100 μl of PBMC was added to 100 μl *Pf*AMA1 (30, 6 or 1.2 μg/ml in PBS) in 96 well Nunclon surface flat plates (Life Technology). Plates were incubated (six days, +37°C, humidified 0.5%CO2) before labelling (10 μl ^{3}H thymidine, 0.25 uCi per well, 24 hours) and harvested onto Wallac filter mats using the Wallac Beta plate harvester. Incorporated ^{3}H-thimidine was determined using a Wallac Beta Plate counter. Stimulation indices (SI) were calculated relative to control wells to which no *Pf*AMA1 had been added. PBMC were tested in parallel for their ability to be stimulated with Tetanus Toxoid (Purified Tetanus Toxoid 150 Lf/ml, RIVM, Bilthoven, The Netherlands) and phytohaemagglutinin 5 μg/ml (PHA-L, Sigma-Aldrich).

In vitro parasite growth inhibition

Antibodies to be used for parasite inhibition assays were purified on protein A columns (Immunopure Plus Pierce, St Louis, MO, USA) using standard protocols, exchanged into RPMI 1640 using Amicon Ultra-15 concentrators (30 kDa cutoff, Millipore, Ireland), filter-sterilised and stored at −20°C until use. IgG concentrations were determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

P. falciparum strain FCR3 was cultured *in vitro* using standard *P.falciparum* culture techniques in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. FCR3 AMA1 (accession no. M34553) differs by one amino acid in the pro-sequence from FVO AMA1 (accession no. AJ277646).

The effect of purified IgG antibodies on parasite invasion was evaluated with two IgG concentrations (5 and 10 mg/mL, respectively) in triplicate using 96 well flat-bottomed plates (Greiner) with synchronized cultures of *P. falciparum* schizonts at a starting parasitemia of 0.2–0.4%, a haematocrit of 2.0% and a final volume of 100 μL containing 10% control non-immune human serum, 20 μg mL^{-1} gentamicin in RPMI 1640. After 40 to 42 hours, cultures were resuspended, and 50 μL was transferred into 200 μL ice-cold PBS. The cultures were then centrifuged, the supernatant removed and the plates were frozen. Inhibition of parasite growth was estimated using the pLDH assay as previously described (14). Parasite growth inhibition, reported as a percentage, was calculated as follows: $100-((Od_{experimental}-Od_{background})/(Od_{control}-Od_{background})\times100)$. IgG purified from plasma before immunisation was used as a control, and culture medium was used to measure the background Od.

Statistical methods

Safety analyses were based on intention to treat data selection ($n=56$). For immunology assays, per protocol analyses were used ($n=47$). Between group differences were calculated by one-way ANOVA, using post-hoc Bonferroni when $p<0.05$. Differences between high and low dose groups were compared with Mann-Whitney U test.

Results

Study population

Participants were recruited at the Radboud University Nijmegen Medical Centre from September to October 2005. Of 92 adult males screened in and having provided informed consent, 56 were eligible and enrolled (fig. 1). Main reasons for exclusion were abnormal laboratory parameters or unable to be closely monitored for social, geographic or psychological reasons. Table 1 shows the demographics of volunteers per randomised group. The mean age was 23 years old (range 18–42 years) and all but one were Caucasian.

Safety and reactogenicity

No serious adverse events occurred that were definitely, probably, or possibly related to immunisation. No clinically relevant changes in vital signs or laboratory values were reported throughout the study. Forty-seven volunteers (84%) received all three immunisations; nine were excluded for one or more immunisations (fig. 1). Two of these were excluded for reasons unrelated to the trial procedures. One (Alhydrogel™ 10 μg group) developed a generalised rash assessed as unrelated to the vaccine between the first and second immunisations and one (Montanide 10 μg group) received a concomitant hepatitis B immunisation. Seven volunteers were excluded because they developed grade 3 erythema (diameter >50 mm) after the second immunisation; one in the Montanide 10 μg group, the other six in the AS02 groups (two in the 10 μg group, four in the 50 μg group).

Volunteers in all groups presented with local injection site reactions, the most predominant being transient mild to moderate pain (80–100%, table 2). Erythema was commonly observed (10 of 17 volunteers) in the AS02 adjuvanted groups, occurring after the second and third immunisation. In the Montanide group 4 of 18 volunteers developed erythema. Seven volunteers reported grade 3 erythema and were withdrawn from further immunisation after dose 2. The skin in grade 3 (diameter >50 mm) erythema was not painful and did not limit daily activities. Episodes of erythema generally lasted 2–3 days.

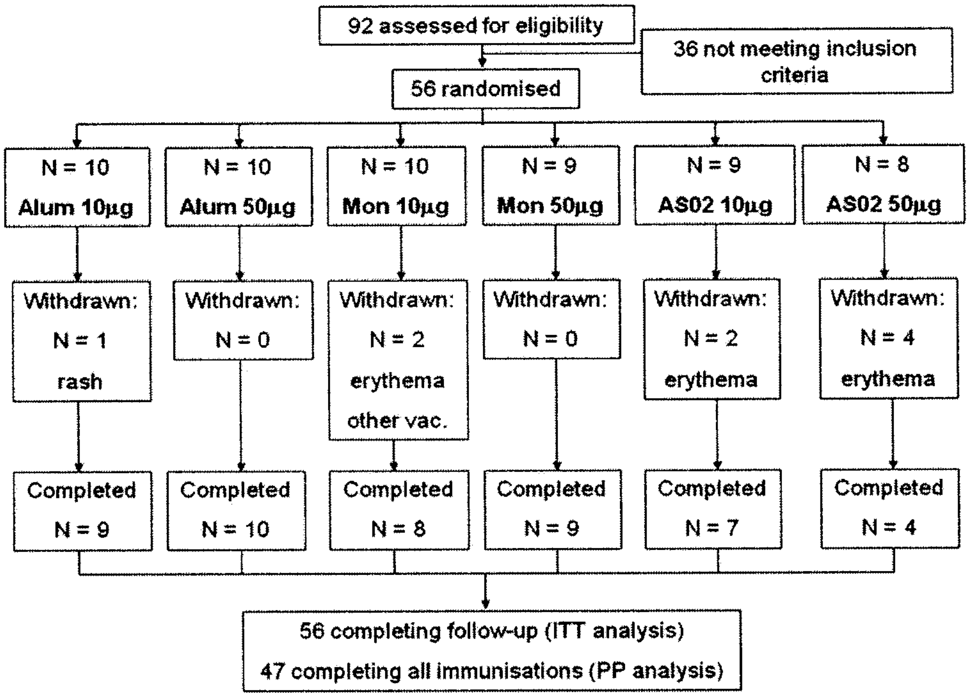


Figure 1. Study flow chart showing number of volunteers randomised, withdrawn and completing follow-up. Coding for adjuvant as follows: Alum = *Alhydrogel*™, Mon = Montanide. Reasons for withdrawal are given: "rash" = allergic rash unrelated to study procedure, "erythema" = grade 3 injection site erythema leading to withdrawal, "other vac." = concomitant Hepatitis B vaccination leading to exclusion.
doi:10.1371/journal.pone.0003960.g001

Induration at the site of injection occurred in three volunteers in the course of the study (table 2). One volunteer, in the Montanide 10 µg group, developed moderate induration 15 days after the first immunisation, lasting for five days. The second and third immunisations in this volunteer were well tolerated; induration did not re-appear. Another volunteer developed induration starting nine days after the first immunisation in the left arm with 50 µg *Pf*AMA1 in Montanide, lasting 25 days. The second immunisation was well tolerated, but the left arm induration re-appeared one day after the third immunisation, accompanied by pain and induration at the previous immunisation site in the contralateral (right) arm. Four weeks later, the induration became soft and fluctuated, indicating abcess formation. A total of 63 ml of opaque, brown fluid was aspirated by two subsequent punctures, after which the abscess and induration resolved spontaneously and disappeared completely at 81 days post third immunisation. The third volunteer, also in the 50 µg *Pf*AMA1 adjuvanted with Montanide group, developed moderate induration nine days after the second immunisation which lasted approximately one week. Six days after the third immunisation he developed induration at his left arm (the site of the first and last immunisation) which eventually started fluctuating. A total of 130 ml brown, opaque fluid was collected by means of two punctures. Thereafter, spontaneous percutaneous drainage occurred and the lesion resolved 57 days after the third immunisation. Both volunteers did not have any systemic symptoms such as fever during this time period. Abcesses were only mildly painful, but limited volunteers daily activities because of their size.

Table 1. Demographic data, race and age of volunteers per dose and adjuvant group.

Adjuvant		Aluminum		Montanide		AS02		All
*Pf*AMA1 dose		10 µg	50 µg	10 µg	50 µg	10 µg	50 µg	
N		10	10	10	9	9	8	56
Race	Caucasian	10	10	10	9	8	8	55
	Oriental	-	-	-	-	1	-	
Age (years)	Mean	22.4	22.6	22.5	22.6	24.1	24.4	23.0
	STD	3.1	2.0	4.5	3.8	7.3	6.1	4.6
	Minimum	18	19	18	19	19	18	18
	Maximum	29	26	33	31	42	36	42

doi:10.1371/journal.pone.0003960.t001

The aspirated fluids from both volunteers were abundant in red blood cells and lymphocytes with low Creatinine Kinase (CK) levels. Repeated cultures did not reveal any bacterial contamination. Serum CK levels were normal. Circulating levels of C-reactive protein remained below detection levels (indicating that the reaction was a local response). Ultrasound examination suggested an intramuscular and subcutaneous localisation of the fluid-filled cavity.

Systemic reactions were infrequent in the Alhydrogel™ and Montanide groups and occurred mainly in the AS02 groups. The systemic adverse events occurred within 24 hours of immunisation and usually resolved within two days. The most prevalent systemic adverse events were headache (77.8–87.5%) and malaise (66.7–87.5%) in the AS02 groups. Four of those volunteers reported grade 3 headache or malaise. Most of the systemic adverse events occurred after dose 2. There was no effect of antigen dose on reactogenicity. No changes in blood pressure were noted in any of these volunteers.

Table 2. Number of volunteers reporting vaccine related adverse events per dose and adjuvant group.

Adjuvant	Alum		Montanide		AS02		Total
*Pf*AMA1 dose	10 µg	50 µg	10 µg	50 µg	10 µg	50 µg	
N	10	10	10	9	9	8	56
Total	8 (80.0%)	10 (100%)	9 (90.0%)	9 (100%)	9 (100%)	8 (100%)	53 (94.6%)
LOCAL							
Pain	8 (80.0%)	10 (100%)	8 (80.0%)	9 (100%)	9 (100%)	8 (100%)	52 (92.9%)
Erythema	-	-	2 (20.0%)	2 (22.2%)	4 (44.4%)	6 (75.0%)	14 (25%)
Swelling	-	-	1 (10.0%)	-	3 (33.3%)	1 (12.5%)	5 (8.9%)
Induration	-	-	1 (10.0%)	2 (22.2%)	-	-	3 (5.4%)
Sterile abscess	-	-	-	2 (22.2%)	-	-	2 (3.6%)
SYSTEMIC							
Headache	1 (10.0%)	-	2 (20.0%)	-	6 (66.7%)	7 (87.5%)	16 (28.6%)
Malaise	-	-	-	1 (11.1%)	6 (66.7%)	7 (87.5%)	14 (25.0%)
Fever	-	-	-	-	5 (55.6%)	5 (62.5%)	10 (17.9%)
Myalgia	-	-	-	-	4 (44.4%)	2 (25.0%)	6 (10.7%)
Nausea	1 (10.0%)	-	-	-	1 (11.1%)	2 (25.0%)	4 (7.1%)
Fatigue	-	-	-	-	-	2 (25.0%)	2 (3.6%)
Arthralgia	-	-	-	-	1 (11.1%)	-	1 (1.8%)
Abdominal pain	-	-	-	-	1 (11.1%)	-	1 (1.8%)

doi:10.1371/journal.pone.0003960.t002

Humoral immune response

Peak antibody titers were observed one month after the final immunisation. 100% of volunteers in the 10 and 50 µg AS02 and 50 µg Montanide groups showed a greater than four-fold increase in antibody titer over pre-immunisation compared to 60% in the 10 µg Alhydrogel™, 80% in the 50 µg Alhydrogel™ and 90% in the 10 µg Montanide groups (fig. 2). All vaccinees had reached IgG titers comparable to or higher than semi immune sera. Two and four months post final immunisation both AS02 groups and the Montanide 50 µg group showed the highest IgG titers but given the small sample sizes there was no power to detect statistical differences between groups. Antibody titers decreased further one year post immunisation, with the steepest decline being in the Montanide groups, to a level comparable with the reference Alhydrogel™ groups. One year post vaccination, titers in the 10 µg AS02 group were significantly higher as compared to the reference group (post hoc Bonferroni when compared with low dose Alhydrogel™ reference group $p<0.01$, 95% CI 0.25 to1.5). Vaccinees receiving 50 µg PfAMA1 generally showed a trend towards higher antibody titers than the corresponding 10 µg group, except for the AS02 groups where antibody titers were not antigen dose-dependent.

Sera from vaccinees could be shown to recognise the native *Pf*AMA1 by immunofluorescence in a dose dependent manner. Eight of fifteen samples were positive in IFA, amongst which were four samples with the highest antibody titers. The staining pattern found in positive samples localised to the same structures as 4G2 rat monoclonal antibody (fig. 3).

Cellular immune response

In all groups, induction of IFNγ and IL-5 cytokines could be demonstrated (fig. 4). The magnitude of cytokine production was not dose dependent or dependent on the number of immunisations. Rather, IFNγ production in many samples decreased after the third immunisation. For both cytokines, *Pf*AMA1 induction was comparable or higher than that following stimulation with 5 µg Tetanus Toxoid (data not shown). Cytokine production in the different groups did not differ significantly from each other (for INFγ $p = 0.18$, for IL-5 $p = 0.14$). Ratio's of IFNγ / IL-5 production were also not significantly different between adjuvant groups (data not shown), but showed a trend towards higher ratio in the Montanide and AS02 groups (Day 84 mean ratio Alhydrogel: 1.16 (95% CI: 0.08 to 2.23), Montanide: 2.82 (95% CI: 1.44 to 4.21), AS02: 2.66 (95% CI: 0.57 to 4.76)).

All groups showed Peripheral Blood Mononuclear Cell proliferation upon stimulation with *Pf*AMA1 (fig. 5). Stimulation indices between PBMC's stimulated with 30, 6 or 1.2 µg/ml *Pf*AMA1 were similar. All groups of volunteers showed significant increase in proliferation upon stimulation with *Pf*AMA1 after the second immunisation. After the third immunisation none of the groups showed a further increase in stimulation index, rather the 10 µg Montanide group showed a significant decrease after the third immunisation ($p = 0.03$). There were no significant differences in stimulation index between the different adjuvant or dose groups.

In vitro parasite growth inhibition

To estimate functionality of the induced antibodies, an *in vitro* GIA was performed. Results are shown as percentage inhibition compared to pre-vaccination sera from the same individual (fig. 6). At a concentration of 10 mg/ml, the median growth inhibition in the Montanide 50 µg and AS02 groups was about 30% and 50% respectively. In the Alhydrogel™ and Montanide 10 µg groups median inhibition was lower, ranging from 4 to 17%. Only differences between Alhydrogel™ and AS02 groups were significant ($p = 0.002$).

Discussion

This trial demonstrates that reactogenicity of *Pf*AMA1-FVO[25-545] varies, depending on the adjuvant. Immunogenicity

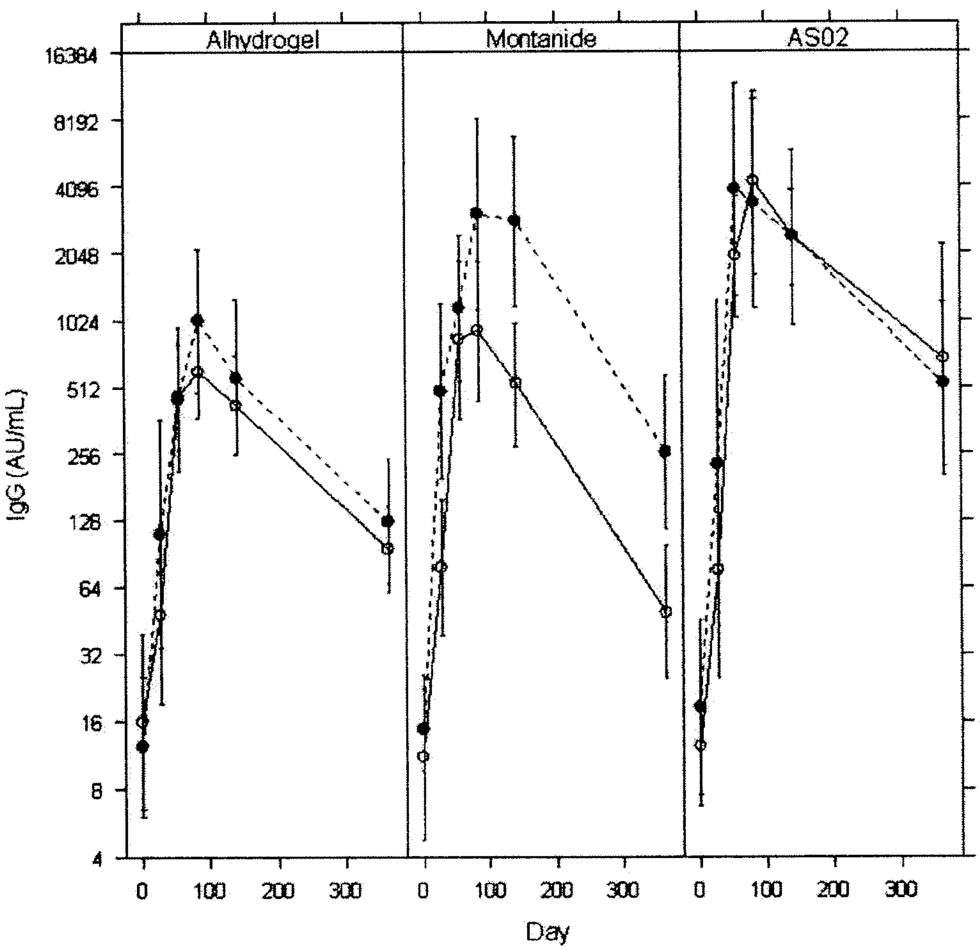


Figure 2. Mean log anti-AMA-1 titers with standard error of the mean for low and high dose per adjuvant group. Anti-AMA-1 titers were determined by ELISA for the six different groups, immunized with *Alhydrogel*™, Montanide and AS02 adjuvanted *Pf*AMA1 vaccine. Dashed lines represent high dose of *Pf*AMA1 (50 µg), continuous lines represent low dose groups (10 µg). Measurements were performed at baseline, 28 days after the first, second and third immunisation (day 28, 56 and 84 respectively) and day 140 and 365.
doi:10.1371/journal.pone.0003960.g002

at both high and low doses and in all adjuvant formulations is good, although the type and magnitude of immune response varied among different adjuvant groups.

*Pf*AMA1-FVO[25-545] mixed with the adjuvants *Alhydrogel*™, Montanide and AS02 tended to be locally reactogenic, mainly causing short lasting injection site pain when administered to

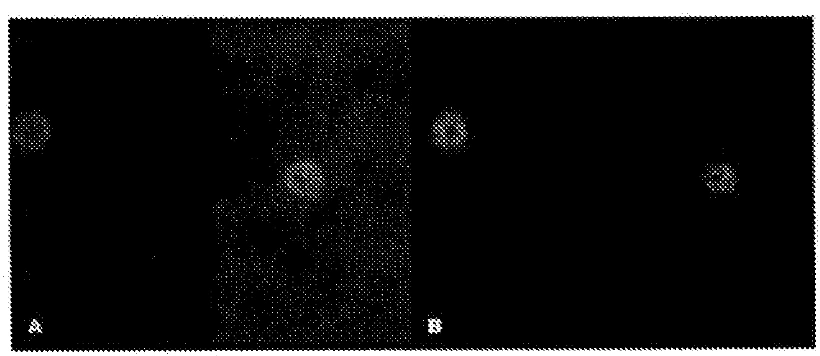
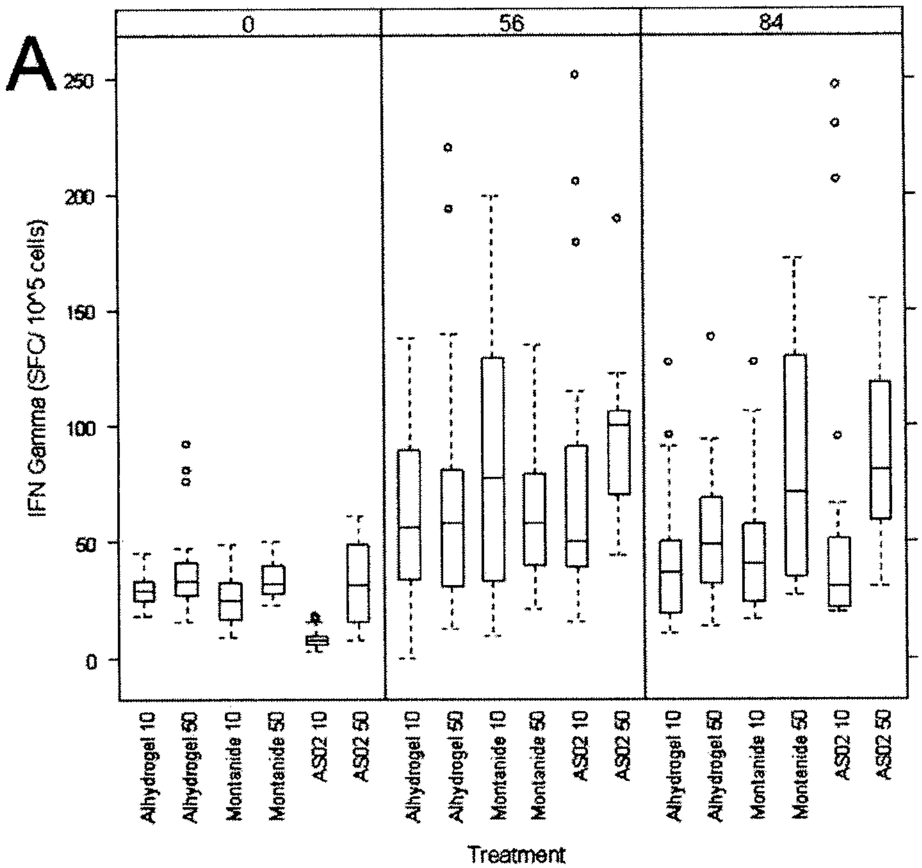
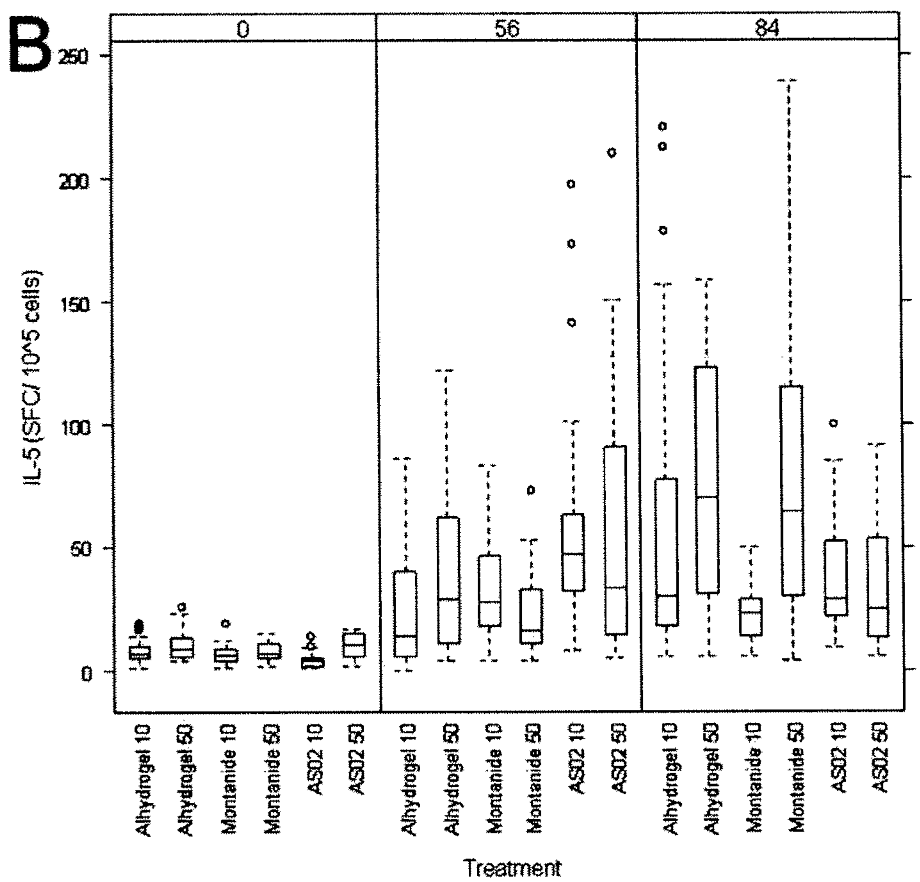


Figure 3. Representative immunofluorescent microscopy picture, showing recognition of native antigen on merozoites by induced anti-AMA1 antibodies. Immunofluorescence picture of merozoites incubated with 40× diluted anti-AMA1 plasma from an immunized volunteer one month after final immunisation stained with Rabbit anti-human immunoglobulin FITC (A) and DAPI (B). Photo was taken at magnification 400×. Incubation with monoclonal antibody 4G2, a pan-specific anti-AMA1 antibody, confirmed the surface staining pattern (not shown).
doi:10.1371/journal.pone.0003960.g003


A
0
56
84
IFN Gamma (SFC/ 10^5 cells)
0
50
100
150
200
250
Alhydrogel 10
Alhydrogel 50
Montanide 10
Montanide 50
AS02 10
AS02 50
Treatment


B
0
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IL-5 (SFC/ 10^5 cells)
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200
250
Alhydrogel 10
Alhydrogel 50
Montanide 10
Montanide 50
AS02 10
AS02 50
Treatment

Figure 4. ELISPOT assay for IFNγ (A) and IL-5 (B) after stimulation with 6 µg *Pf*AMA1. Peripheral Blood Mononuclear Cells from immunised volunteers 28 days after the second immunisation and 28 days after the third immunisation (day 56 and 84 respectively) were stimulated with 6 µg of *Pf*AMA1 vaccine. Production of IFNγ and IL-5 was measured by counting spots in ELISPOT plates. Box plots and whiskers show the range and the 25th, 50th and 75th percentile of spots per 2×10^5 cells. Circles represent outliers.
doi:10.1371/journal.pone.0003960.g004

healthy adult volunteers. Most post immunisations adverse events were mild-to-moderate in intensity and have been seen previously with other vaccines[35–40]. Because this was the first time *Pichia Pastoris* produced FVO *Pf*AMA1 antigen was being given to humans, the occurrence of a grade 3 adverse event was a stopping criterium, which led to withdrawal of seven subjects post dose 2 for grade 3 (>50 mm) erythema. However, the erythema observed resolved spontaneously within three days of onset without any sequelae. The erythema is not considered a hindrance for further vaccination with the AS02 adjuvant. In terms of systemic adverse events, most were related to the AS02 adjuvant and transient resolving within two days with no sequelae. The pattern of transient, primarily mild to moderate systemic adverse events has been reported with another AMA-1 antigen adjuvanted with AS02 [41].

Three immunisations with 50 µg *Pf*AMA1 adjuvanted by Montanide induced a sterile abscess in two of ten volunteers. Progression of induration to a sterile abscess has been previously reported before after immunisation with Montanide [42–44]. In all reports the development of an abscess followed intramuscular immunisation and was accompanied by enhanced immunogenicity. The increased reactogenicity of Montanide-adjuvanted vaccines has been attributed to a combination of antigen dose and the formation of a vaccine depot that may persist locally and that is inherent to water-in-oil emulsions [45]. Similarly, induration at the previous immunisation site has been attributed to persistent antigen in previous trials [46]. A less condensed vaccination regimen and avoidance of the same injection sites may be measures to avoid induration.

To date, there are four other reports on clinical phase Ia trials of a *Pf*AMA1 vaccine. These trials employed different *Pf*AMA1 constructs and utilized different adjuvants. The constructs were of *P. pastoris* or *E. coli* origin or used a virally vectored delivery system [47–50].

The *P. pastoris*-produced *Pf*AMA1 comprised recombinant proteins based on sequences from the ectodomains of FVO and

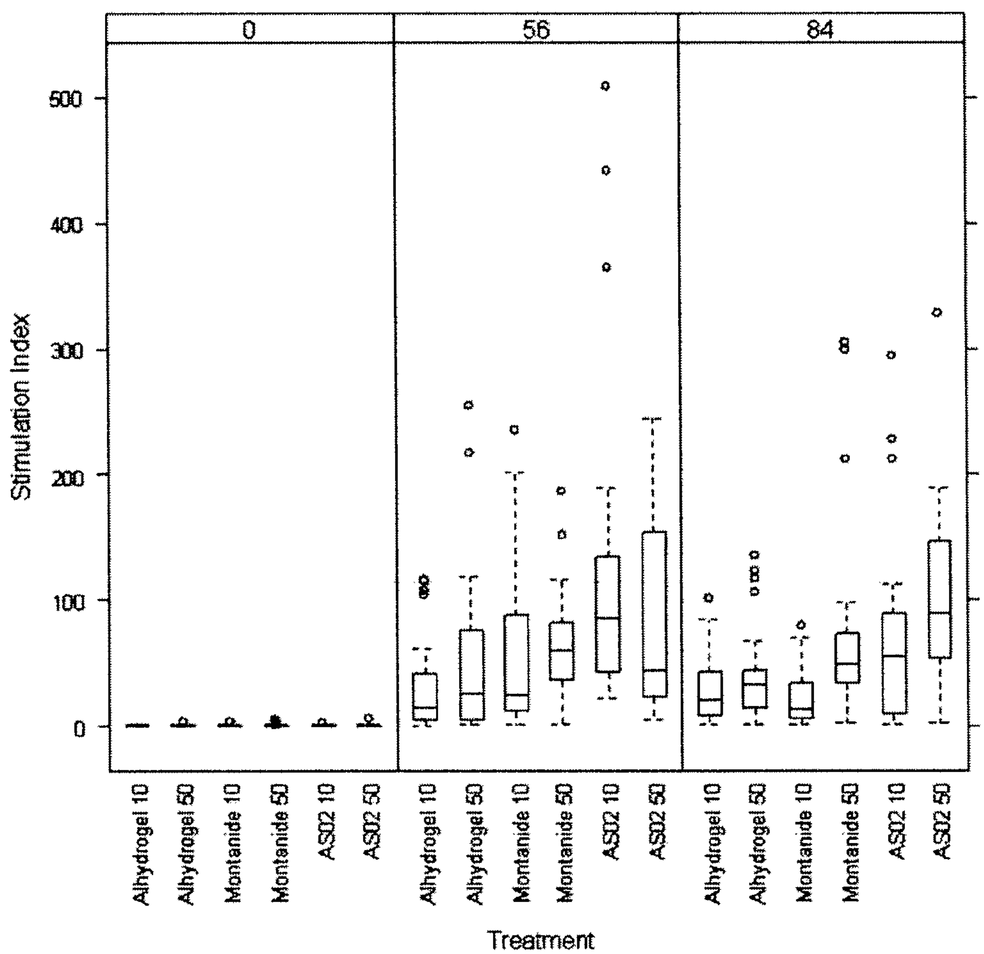

Figure 5. Stimulation indices in response to 6 µg/ml *Pf*AMA1 presented as box plots and whiskers. Peripheral Blood Mononuclear Cells from immunised volunteers 28 days after the second immunisation and 28 days after the third immunisation (Day 56 and 84 respectively) were stimulated with 6 µg of *Pf*AMA1 vaccine. Cell proliferation was measured by adding ^{3}H thymidine and calculated relative to control wells. Box plots and whiskers show the range and the 25th, 50th and 75th percentile. Circles represent outliers. Measurements were performed at baseline, 28 days after the second immunisation and 28 days after the third immunisation (day 56 and 84 respectively).
doi:10.1371/journal.pone.0003960.g005

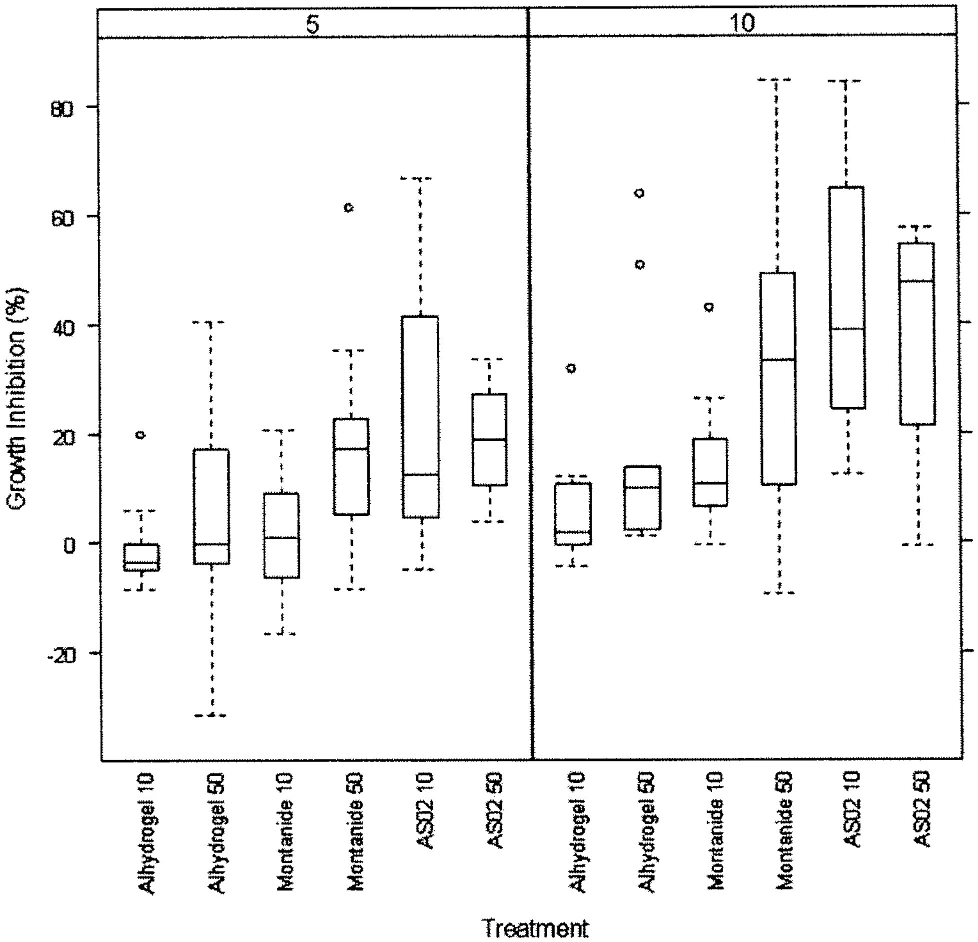

Figure 6. Percentage of growth inhibition of FVO-strain *P. falciparum* parasites after addition of 5 or 10 mg/ml IgG. Serum samples from volunteers immunised with *Pf*AMA1 were obtained four weeks after the final immunisation by per protocol analysis and included in a merozoite growth inhibition assay. Growth inhibition is expressed as a percentage to control.Boxes show 25th, 50th and 75th percentile growth inhibition, whiskers show the range, circles are outliers.
doi:10.1371/journal.pone.0003960.g006

3D7 strain AMA1 adjuvanted with *Alhydrogel*^{TM} have been tested both in a phase Ia and Ib trial. As with previous studies in which malarial antigens have been adjuvanted with *Alhydrogel*^{TM}, this candidate vaccine showed an acceptable reactogenicity profile but a limited immune response. The Malkin et al. phase Ia trial shows a GIA response in only 4 of 22 subjects despite high seroconversion rates [51], similar to the data obtained here with *Alhydrogel*^{TM}. Interestingly, in our study, the *P. pastoris PfAMA1* combined with *Alhydrogel*^{TM} was much less reactogenic and did not produce any erythema or induration, even though the doses were comparable. The lower Alhydrogel dose (500 µg per immunisation) used in this trial, as compared to Malkin et al. (800 µg) may also play a role in its decreased reactogenicity.

There are two trials utilizing the *E. coli*-produced 3D7 strain AMA1, one reconstituted in Montanide and a second formulated in AS02. The AMA1-Montanide combination was considered safe but the trial was compromised by apparent loss of potency [52]. The AMA1-AS02 combination [53] showed comparable local and systemic reactogenicity. Although Polhemus et al. were able to show recognition of the native antigen by IFA, growth inhibition results were approximately two fold lower than those found in this study.

Lastly, *Pf*AMA1 has also been evaluated in a multi-antigen malaria vaccine delivered in an attenuated vaccinia virus. Although weak protective effects were found, immunogenicity in that trial was poor [54].

After one year follow-up, we found antibody levels still to be significantly higher than baseline for all groups. This is in sharp contrast to the results of Malkin et al. [55] who reported detectable antibodies in only 50–90% of volunteers by day 364, even though they had been boosted much later (on Day 180). This suggests that a more condensed immunisation regime may affect the persistence of antibodies.

In this trial we have shown that the combination of clinical grade *Pf*AMA1 FVO [25-545] *P. pastoris* expressed material with either Montanide or AS02 is significantly more immunogenic than previous *Pf*AMA1 formulations, being capable of inducing high levels of antibodies for both dosages in both adjuvant groups. A positive trend between antigen dose, antibody response and *in vitro* parasite growth inhibition could be detected, although the effect of antigen dose on immunogenicity was negligible compared to the effect of varying the adjuvant. The wide variety of immune responses found in different adjuvant formulations stresses the importance of adjuvants as a critical component in malaria vaccine development.

The functionality of vaccine induced antibodies was assessed by growth inhibition assay. Although this assay has not been validated

as a correlate of protection, this trial demonstrates that the standardised assay is able to demonstrate recognition of the native protein and thus functionality in vitro.

Different adjuvants are known to prompt immune responses towards Th1 or Th2. It has been previously reported that AS02 induces an immune response skewed towards Th1 [56], with production of primarily IFNγ. In contrast *Alhydrogel*™ is known to be Th2 inducer [57]. In this study, ratio's of cytokine production at day 84 showed relatively more IFNγ over IL-5 production in the Montanide and AS02 groups suggesting a pro-Th1 response, although statistically non-significant. Interestingly, the additional third immunisation generally did not lead to a further increase in IFNγ or IL-5 production or in lymphocyte proliferation. Rather, many volunteers showed a reduction in the response after the third immunisation. This difference could not be explained by inter-test variability. It remains to be investigated if it indicates a shift in the relative balance between immediate effector cells and long-lived memory cells.

Although this phase I trial is limited with respect to the size and generalizibility to the target population, it met its objectives to outline a generalizable safety profile. Specifically the direct comparison of the safety profile of different adjuvants is valuable for future development of AMA1 and other malaria vaccines. Furthermore, the malaria vaccine candidate AMA1 provides the possibility of assessing functionality of the immune response by a parasite growth inhibition assay. However, it must be noted that the growth inhibition assay is not validated as a correlate of protection, and is as such a limited predictor for efficacy.

With this study we have shown that the *Pf*AMA1 vaccine combined with different adjuvants, *Alhydrogel*™, Montanide and AS02 provided distinct reactogenicity profiles. All vaccine formulation were immunogenic at both dosages. Growth inhibition results indicate that induction of functional immune responses is probably dependent on adjuvant, underscoring the need for strong immunopotentiators for malaria vaccines. Altogether, these results are promising for a future development of a *Pf*AMA1 malaria vaccine.

Supporting Information

Checklist S1 CONSORT Checklist
Found at: doi:10.1371/journal.pone.0003960.s001 (0.06 MB DOC)

Protocol S1 Trial Protocol
Found at: doi:10.1371/journal.pone.0003960.s002 (0.70 MB PDF)

Amendment S1
Found at: doi:10.1371/journal.pone.0003960.s003 (0.26 MB PDF)

Amendment S2
Found at: doi:10.1371/journal.pone.0003960.s004 (0.42 MB PDF)

Amendment S3
Found at: doi:10.1371/journal.pone.0003960.s005 (0.30 MB PDF)

Amendment S4
Found at: doi:10.1371/journal.pone.0003960.s006 (0.25 MB PDF)

Amendment S5
Found at: doi:10.1371/journal.pone.0003960.s007 (0.52 MB PDF)

Amendment S6
Found at: doi:10.1371/journal.pone.0003960.s008 (1.06 MB PDF)

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Author Contributions

Conceived and designed the experiments: EJR HEB OL SJ OOA BWF WRB JC MCD AVdV AWT RWS. Performed the experiments: MR EJR ECCdJ CH MA VW KT WR QdM AVdV. Analyzed the data: MR CH EBI. Contributed reagents/materials/analysis tools: EJR CH OOA BWF CHK SA. Wrote the paper: MR EJR CH EBI OOA MCD AVdV AWT RWS.

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GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives

Nathalie Garçon†, Patrick Chomez and Marcelle Van Mechelen

The need for potentiating immune responses to recombinant or subunit antigens has prompted GlaxoSmithKline (GSK) Biologicals to develop various Adjuvant Systems for the design of prophylactic and therapeutic vaccines. Adjuvant Systems are formulations of classical adjuvants mixed with immunomodulators, specifically adapted to the antigen and the target population. They can activate the appropriate innate immune system and subsequently impact on adaptive immune responses. AS04 is an Adjuvant System that has demonstrated significant achievements in several vaccines against viral diseases. AS02, another Adjuvant System, is being evaluated in various contexts, where a strong T-cell response is needed to afford protection. Likewise, AS01 has been developed for vaccines where the induction of a yet stronger T-cell-mediated immune response is required. Altogether, the promising clinical results strongly support the concept of Adjuvant Systems and allow for further development of new vaccines, best adapted to the target population and the immune mechanisms of protection.

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Vaccines represent one of the safest and most cost-saving medical advances that have ever been developed in healthcare history. They have allowed the eradication of infectious diseases, such as smallpox, and dramatically reduced the morbidity and mortality due to numerous diseases over the past two centuries (FIGURE 1).

The first generation of vaccines was based on replicating or nonreplicating attenuated pathogens or on whole inactivated microorganisms. The second generation of vaccines used partially purified pathogen-derived antigens, often combined with aluminum salts. Improved production methods, with the objective of increasing the safety profile of vaccines, have led to the development of a third generation of vaccines, based on highly purified subunit antigens and/or antigens produced by recombinant DNA technology. More than two decades ago, GlaxoSmithKline (GSK) initiated the development of recombinant adjuvanted vaccines.

The use of highly purified antigens has decreased the risk of vaccine toxicity but, as a consequence, the immunogenicity of some of these vaccine antigens is suboptimal. Parallel to this development, the understanding of the immune system, particularly the respective impact of the innate and adaptive immunity and their close interaction (FIGURE 2), has allowed for a more rational approach in the design of new vaccines. The evolving understanding of the importance of cell-mediated immunity in the protection against intracellular pathogens (viral, bacterial or parasitic) has substantiated the need for an immune response beyond antibody production and B-cell memory in order to prevent disease. However, this cannot always be elicited adequately by recombinant/subunit antigens alone. Therefore, new strategies have been designed and tested to address this challenge, among them the development of more adequate, target-tailored adjuvants.

For almost 80 years, adjuvants have been known for their ability to increase the immune response against a given antigen, as first demonstrated by Ramon in 1926 [1] and later by Glenny [2], who was the first to use aluminum salts as adjuvants for vaccines. For

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some applications, however, aluminum salts have proved to be of limited use, particularly when there is a need to induce a strong cell-mediated immune response. More specifically, the need for more potent and tailored adjuvants has become crucial in order to: better target the effector response (humoral and cellular); induce long-term persistence of protection with a higher level of immune response, as well as an improvement of the immune memory; overcome a weakened immunity, as seen in immunosenescence, an age-associated immune deficiency, and in immunosuppression; and to allow for immunomodulation.

Improvements in immunological and biochemical tools have made it possible to select among classical and new adjuvants, to use them alone or in combination with the potential to act synergistically. Adjuvants can, therefore, be optimally adapted to the target population and to the known or suspected mechanism of protection against specific infectious diseases.

Adjuvant Systems

Adjuvant Systems are a technology that GSK has been developing for nearly two decades. Adjuvant Systems are based on the combination of classical adjuvants, such as aluminum salts, oil-in-water (o/w) emulsions, liposomes and immunomodulatory molecules, known to have an impact on the innate and/or adaptive immune responses. They allow for tailored immune responses adapted to the pathogens and to the targeted populations. The challenge for this strategy is to find the best suited combination for an optimally effective and safe formulation in which each part can synergize with the other to drive a more adequate immune response.

Classical adjuvants

Since the work of Glenny, classical adjuvants have been developed over decades and have demonstrated safety and efficacy in humans, as demonstrated by the numerous adjuvanted vaccines that have been registered. Aluminum salts are by far the most widely used adjuvants in vaccines in all age populations to date, with a good safety track record following hundreds of millions of doses administered. Over 80% of the current vaccines contain aluminum salts. Their mechanism of action is not yet fully understood but is believed to be related to a depot effect (persistence of antigen), a local irritation/inflammation and/or a better uptake of the antigen by antigen-presenting cells (APCs). Dendritic cells (DCs) are the most effective APC population to induce activation and proliferation of naive T cells. It is known, however, that aluminum

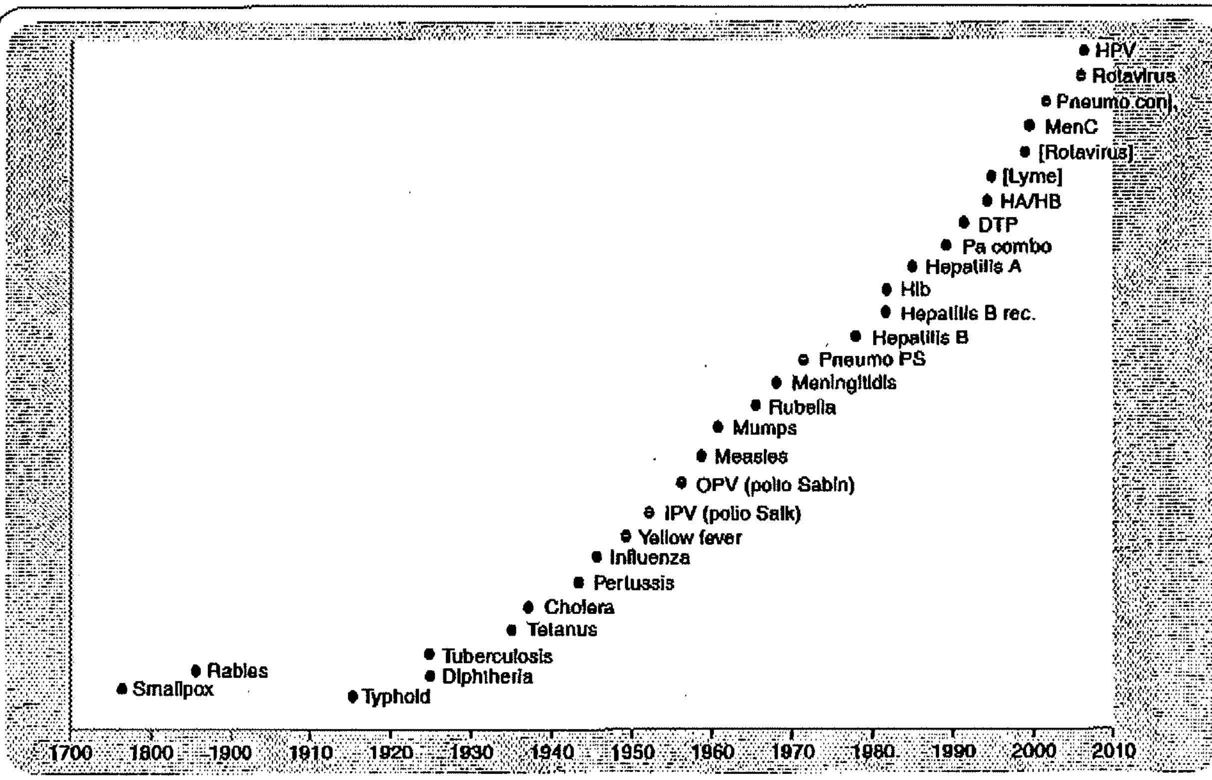

Figure 1. A short history of vaccines. Each dot indicates the date of availability of a vaccine for the disease or the pathogen indicated. Square brackets indicate vaccines that are no longer on the market.
DTP: Diphteria tetanus pertussis; HA/HB: Hepatitis A/hepatitis B; Hepatitis B rec.: Hepatitis B recombinant; Hib: *Haemophilus influenzae* type b; HPV: Human papillomavirus; IPV: Inactivated polio vaccine; MenC: *Neisseria meningitidis* type C; OPV: Oral polio vaccine; Pa combo: Acellular pertussis combinations; Pneumo conj: Pneumococcal conjugate vaccine; Pneumo PS: Pneumococcal polysaccharide vaccine.

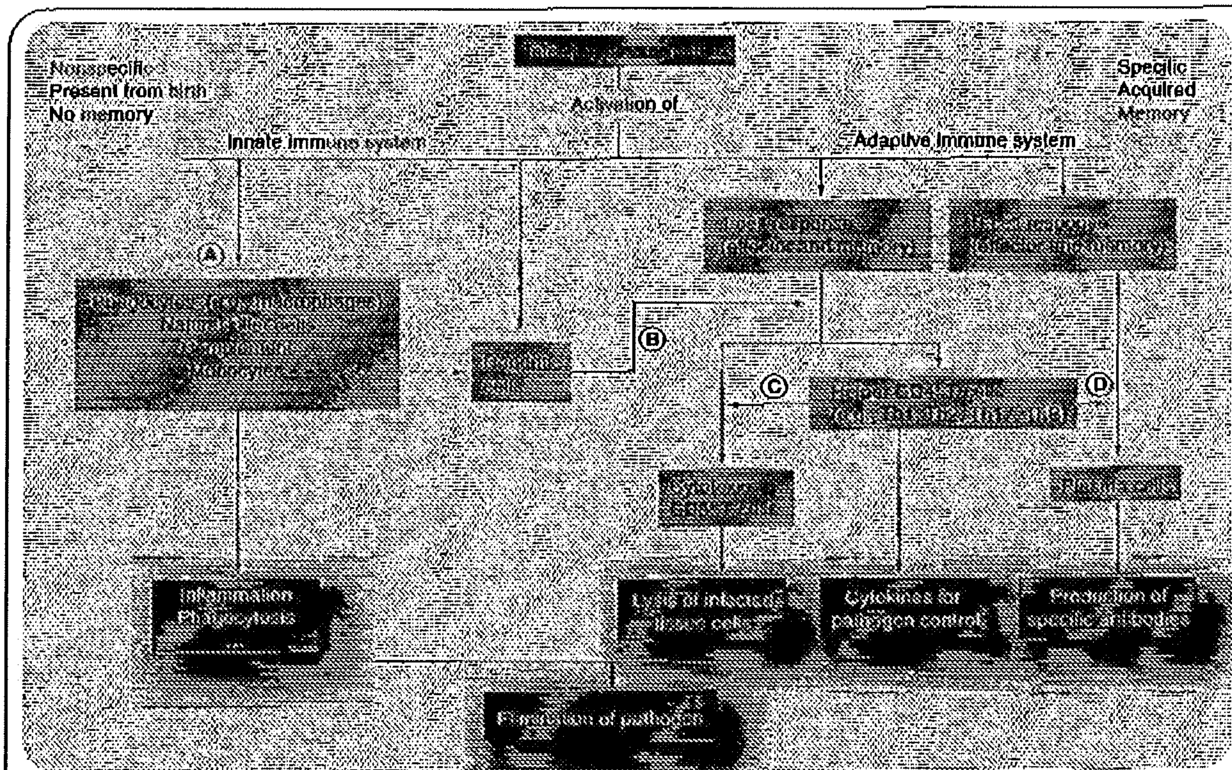

Figure 2. An overview of the immune responses after infection or vaccination. (A) Pathogen components (e.g., DNA and surface proteins) are recognized by various types of receptors, like those of the Toll-like receptor family, at the surface of the effector cells of the innate immunity. **(B)** Dendritic cells are the link between innate and adaptive immunity. The way they are activated by pathogen components will subsequently influence the way they activate T cells. This will end up in different types of adaptive immune responses toward the pathogen, characterized by different subclasses of helper T cells. **(C)** Helper T cells influence the differentiation of cytotoxic T cells. **(D)** Helper T cells influence the differentiation of B cells into plasma cells and, depending on the subclass, determine the type of antibody response toward the pathogen.

salts are poor DC activators and that they do not induce the production of IL-12 [3] and hence may not optimally support the development of Th1 cells.

Emulsions represent another type of classical adjuvants that have been used for the last half century as a means to increase the immune response against a given antigen, in particular by increasing the humoral response [4]. Water-in-oil (w/o) emulsions, such as Freund's incomplete adjuvant (characterized by a discontinuous aqueous phase in a continuous lipid phase), were used in earlier human vaccine candidates, but they were abandoned owing to unacceptable local reactogenicity. This problem may, however, be linked to an insufficient purity of the components in earlier products. Nowadays, more refined w/o emulsions are under consideration for human application [5].

As an alternative to w/o emulsions, o/w emulsions have also been designed and tested for use in human vaccines. O/w emulsions are less reactogenic yet still capable of eliciting a strong humoral response. A vaccine containing such an adjuvant has already been approved for human use (Fluad™ [Novartis, Basel, Switzerland] containing the adjuvant MF59). The mechanism of action of o/w emulsions has yet to be unraveled fully, but is believed to involve innate inflammatory responses, APC recruitment and activation, enhancement of antigen persistence at the injection site and presentation to immune-competent cells, as well as elicitation of different patterns of cytokines. Vaccines containing o/w emulsions have been shown to preferentially induce Th2-type immune responses [6].

Lastly, liposomes have been tested for decades as potential vaccine adjuvants for the delivery of antigens or as immunomodulators [7,8]. Liposomes can be considered as artificial vesicles that comprise an aqueous core enclosed in one or more phospholipid layers. They have been shown to be safe and efficacious, which has led to their registration in a hepatitis A vaccine (Hepaxal™) and an influenza vaccine (Inflexal™ V [Crucell, Leidan, The Netherlands]).

Immunomodulatory molecules

The link between Toll-like receptors (TLRs) and innate and adaptive immunity has been demonstrated within the past 10 years (for a recent review see [9]). Recognition of pathogen-associated molecular patterns is primarily mediated by members

of the TLR family. These receptors are specific for structurally conserved molecules derived from microorganisms that overcome physical barriers, such as the skin or intestinal tract mucosa, and activate immune cell responses in a nonspecific way, acting on the innate immune system. Stimulation of immunologically active cell types results in qualitative and quantitative changes in antigen presentation and cellular activation, hence linking innate and adaptive immunity. Secretion of cytokines and expression of costimulatory molecules induced by engagement of receptors of the innate immune system shape the magnitude and quality of the adaptive immune response. A variety of TLR agonists have been identified and some are being tested and used as vaccine immunomodulators. One of them, 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) (FIGURE 3), is derived from cell wall lipopolysaccharide (LPS) of the Gram-negative *Salmonella minnesota* R595 strain and is detoxified by mild hydrolytic treatment and purification. MPL demonstrates drastically reduced toxicity compared with the parent LPS molecule, while retaining its adjuvant effect. It is a very powerful stimulator of the immune system, known to act as a TLR4 agonist [10,11].

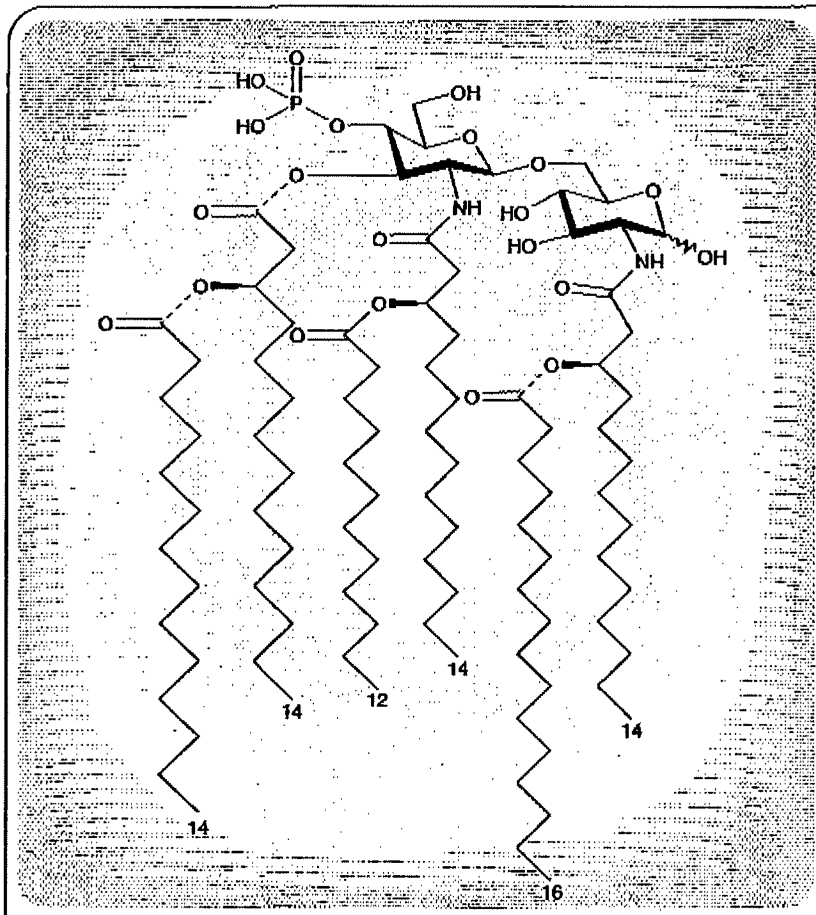

Figure 3. 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL).

Other molecules, not necessarily recognized as TLR agonists, have also been identified as immunomodulators and are being considered as vaccine adjuvants. One of them, QS21 (FIGURE 4), is extracted from the bark of a South American tree, *Quillaja saponaria*. It has been demonstrated to impact the antigen presentation to APCs and to favor induction of cytotoxic T lymphocytes (CTLs) in animal models [12]. Its reactogenicity, however, has hampered its use as an adjuvant. Through appropriate formulation, however, it was possible to eliminate the lytic activity of the molecule, allowing its use in various Adjuvant Systems (FIGURE 5).

The Adjuvant Systems described below are based on the appropriate combination of classical adjuvants (aluminum salt, o/w emulsion and liposomes) and of immunomodulators, such as MPL or QS21.

AS04-formulated vaccines

The Adjuvant System AS04 [13] consists of MPL adsorbed on aluminum hydroxide or aluminum phosphate, depending on the vaccine considered. MPL is a powerful stimulant of the immune system. It acts through binding to TLR4, thereby inducing strong humoral and cellular responses, mostly Th1-biased. AS04 has already been evaluated in various vaccines intended to protect against viral infections/diseases, such as those caused by HBV, human papillomavirus (HPV), herpes simplex virus (HSV), respiratory syncytial virus (RSV) or Epstein-Barr virus (EBV). In total, 30,000 subjects have already been vaccinated with AS04-containing formulations.

Hepatitis B

The HBV is a serious threat for the human population, as persistence of the pathogen in the liver may ultimately lead to chronic liver disease and hepatic carcinoma [14]. An effective hepatitis B vaccine (Engerix™-B [GSK]) adjuvanted with aluminum hydroxide, has been on the market for two decades, providing more than 95% seroprotection in a population under 40 years of age. However, in order to better protect certain population groups that appeared to be low responders to this vaccine, primarily immunocompromised populations, such as hemodialyzed patients, the need remained for a vaccine capable of inducing higher levels of antibody response with a faster onset.

Owing to frequent hemodialysis sessions, chronic exposure to blood products, and because of their immunocompromised status, patients with end-stage renal diseases are at high risk for HBV infections. Classical prophylactic measures consist of

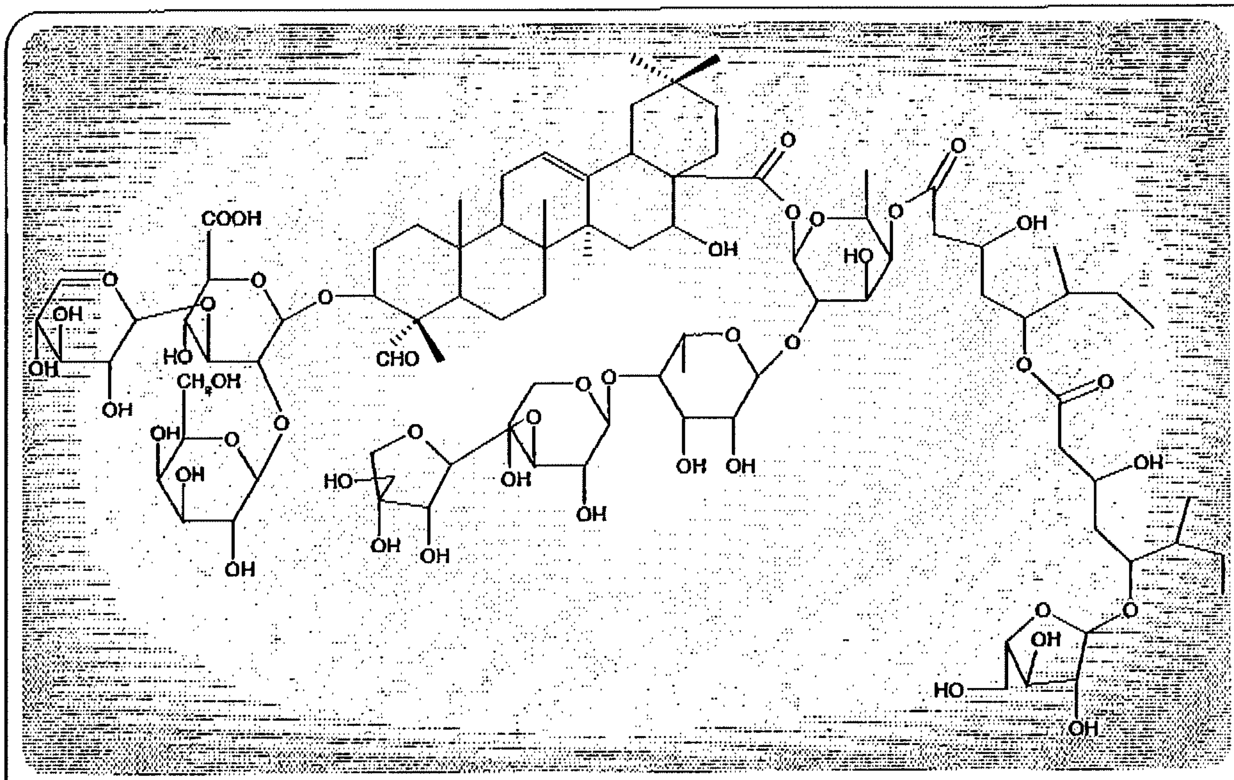

Figure 4. QS21.

repeated injections of a hepatitis B vaccine developed in the mid 1980s. A novel hepatitis B vaccine formulated with AS04 (FENDrix™ [GSK]) has been registered recently and has allowed for the induction of higher specific antibody titers, enhanced cell-mediated responses and increased seroprotection rates with fewer vaccine doses compared with the vaccine adjuvanted with aluminum salts only (FIGURE 6) [15]. The clinical data suggest that protective antibody levels persist longer with the AS04-adjuvanted vaccine [15,16]. FENDrix represents the first AS04-adjuvanted vaccine approved for use in humans.

Human papillomavirus vaccine

Cervical cancer can develop as a result of persistent infection with oncogenic types of HPV [17]. Cervical cancer is one of the leading causes of death due to cancer in women worldwide. The most promising approach for a vaccine is the use of L1 virus-like particles (VLPs) as antigens. These particles are composed of the main component of the viral capsid, L1, produced through recombinant DNA technology, that self-reassemble during the purification process to constitute a VLP devoid of any detectable viral genetic material.

We have developed a vaccine based on L1 VLP technology, containing HPV-16 and -18 type antigens, and formulated with AS04 (Cervarix™ [GSK], recently approved for use in Australia and under review for approval by other regional regulatory authorities). This vaccine was developed to induce a strong and sustained immune response, as manifested by a rapid and vigorous onset of antibody production that persists at a high level over time. Protection against HPV infection is mainly conferred by antibodies in the cervicovaginal mucosa, which are thought to be derived from transudation of serum antibodies. In addition, the AS04-adjuvanted vaccine induces not only high antibody levels but, more importantly, functional antibodies, as evaluated by their long-lasting virus neutralization capacity (FIGURE 7) [18].

GSK's AS04-adjuvanted prophylactic HPV16/18 L1 VLP vaccine has been shown to be highly immunogenic for both HPV-16 and -18 in female subjects aged 10–55 years [19] (MANUSCRIPT IN PREPARATION). In one study in women 15–25 years of age antibody titers remained substantially higher than natural infection titers for up to 5.5 years following vaccination [20] (MANUSCRIPT SUBMITTED). In women 15–55 years old, cervicovaginal mucosal antibodies specific for HPV-16 and -18 persisted at least 1 year after the full vaccination course [GSK data on file]. In a double-blind, randomized, placebo-controlled Phase II clinical trial involving HPV seronegative and DNA-negative participants at study entry, it was observed that the vaccine conferred 100% protection for at least 12 months against persistent infections with HPV-16/18 [20,21]. The vaccine was 100% efficacious in preventing the occurrence of cervical intraepithelial neoplasia (CIN 2⁺) associated with HPV-16/18 infection for up to 5.5 years [22]. In an ongoing Phase III clinical trial, interim analysis after 15 months

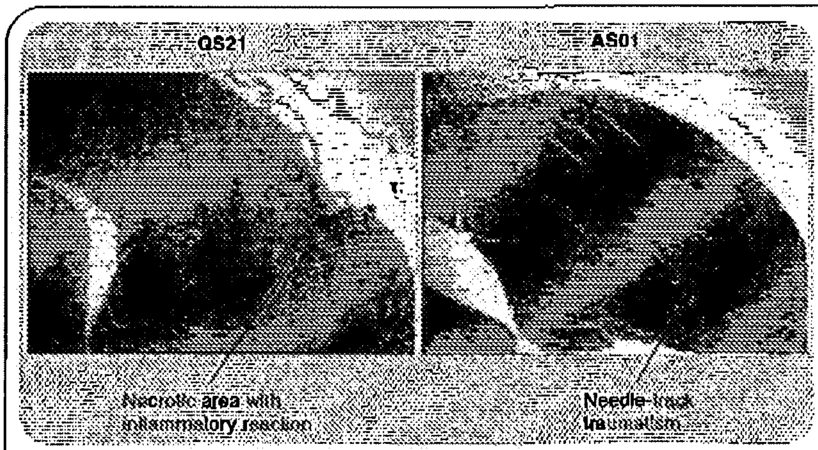

Figure 5. Reactogenicity of QS21 either alone or as a component of AS01. QS21 (5 µg or 50 µl) was injected into the tibialis muscle of a male rat, either alone or formulated in AS01. After 3 days following injection, the rat was sacrificed, the muscle was fixed in formalin and embedded in paraffin. 5 µm transverse sections were stained in hematoxylin–eosin. It is concluded that the formulation of QS21 in an Adjuvant System decreases adverse events in skeletal muscle.

showed up to 100% efficacy against CIN 2+ attributed to HPV 16/18 infections [23]. Substantial vaccine efficacy was also demonstrated against persistent infection with specific nonvaccine oncogenic HPV types 31, 45 and 52 for at least 6 months following vaccination [20,23].

The ability of the AS04-adjuvanted HPV vaccine to afford long-term specific immune memory aims to sustained protection as observed in clinical studies.

Herpes simplex vaccine

HSVs are common human pathogens, with two subtypes, 1 and 2, infecting oral and genital areas. After infection of skin or mucosa, the virus migrates through peripheral nerves to reach dorsal root ganglia where it develops life-long latency. Upon spontaneous reactivation, it migrates back to the skin and mucosa, where it causes painful lesions and is shed, leading to new infections in contacts [24].

Different vaccine approaches have been attempted [25], but as yet, none has been completely successful in preventing infection. Currently, one promising approach makes use of purified recombinant HSV proteins associated with an appropriate adjuvant. As for the HPV vaccine, protection needs to occur at the level of the site of infection, the mucosal tissue, through neutralizing antibodies transudating from the serum, in addition to a strong cell-mediated immunity.

We have developed a candidate vaccine composed of glycoprotein D from HSV-2 (gD2) adjuvanted with AS04. Preclinical studies in the guinea pig model have demonstrated the validity of this approach. Immunization with gD2/AS04 almost completely prevented primary disease with HSV-1 and -2 but not mucosal infection [26]. Cross-protection was probably due to the high degree of conservation of the gD proteins between the two HSV types. Furthermore, it was observed in the same study that guinea pigs immunized with gD2/AS04 were better protected against recurrent disease than were guinea pigs immunized with a gD2 vaccine adjuvanted with aluminum salts alone (FIGURE 8), which suggests that the addition of MPL improved protection against latent infection. Indeed, it was further observed in guinea pigs that viral load in ganglia was reduced by two orders of magnitude after vaccination with gD2/AS04 and that viral shedding into the genital tract was also significantly lower than in control animals [27].

The efficacy of this vaccine was evaluated in Phase III, double-blind, randomized, controlled studies in subjects whose regular sexual partners had a history of genital herpes. Although the vaccine was shown to induce high titers of anti-HSV antibodies, as well as HSV-specific cellular immune responses, in both genders, significant protection (73%) against the disease was observed in women who were seronegative for HSV-1 and -2 before vaccination, but not in men nor in HSV-1-positive women [28]. The protection lasted for at least 2 years. To further assess the efficacy of the

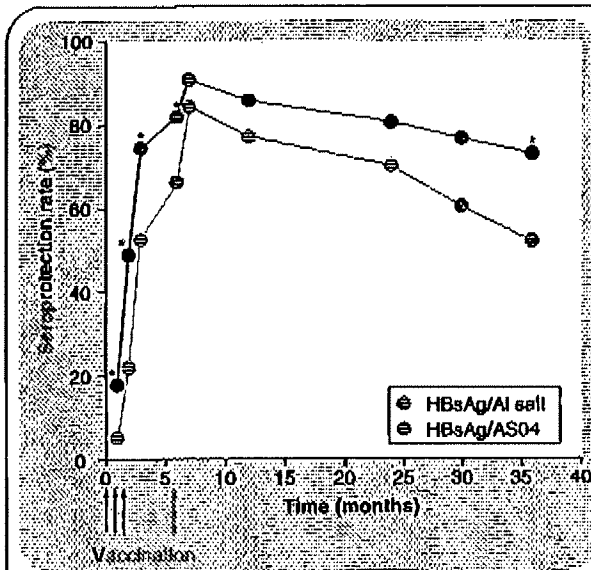

Figure 6. Comparison of the anti-HBsAg seroprotection rates over time induced in humans after vaccination with two different formulations of hepatitis B vaccine. Both vaccines were administered to prehemodialysis/hemodialysis patients (>15 years of age) according to a 0-, 1-, 2- and 6-month immunization schedule. Formulation with AS04 (FENDrix™) allows for the induction of higher specific antibody titers, hence increased seroprotection rate, with less administration of vaccine doses, as compared with the vaccine adjuvanted with aluminum salt only (Engerix™). Seroprotection rate was defined as the percentage of subjects with anti-HBs antibody titers of 10 mIU/ml or higher. Significant differences ($p < 0.05$) between the antibody titers of both vaccine groups are indicated by asterisks. Data from [15].

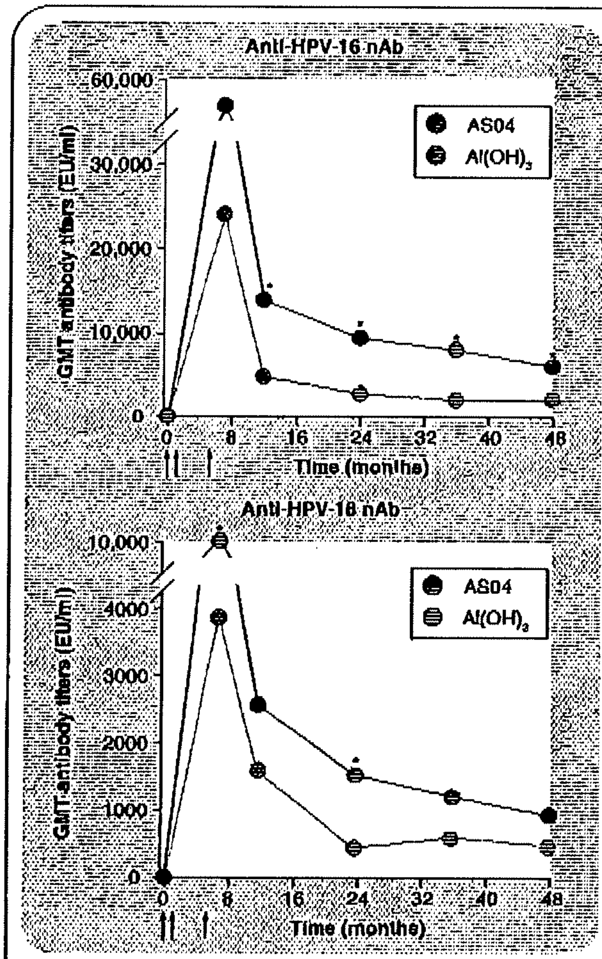

Figure 7. Comparison between AS04 and aluminum salts in the context of human papillomavirus (HPV) vaccine. Human subjects were vaccinated with HPV-16 and HPV-18 L1 virus-like particles adjuvanted with AS04 or with aluminum salt. The levels of HPV-16- and -18-neutralizing antibodies were measured at different time points by a pseudoneutralization assay. Significant differences ($p < 0.05$) between the antibody titers of the AS04 and the aluminum salt group are indicated in the figure by asterisks.
Arrows indicate vaccination.
GMT: Geometric mean titer; nAb: Neutralizing antibody.
Modified from [18].

gD2/AS04 vaccine in HSV-1 and -2 double-negative women, a pivotal Phase III clinical trial enrolling approximately 7000 women between 18 and 30 years of age is ongoing in collaboration with the NIH, and the first results are expected to be available in the coming years.

Respiratory syncytial virus vaccine

RSV is a major cause of respiratory tract illness in infants and children, adults with impaired immunity and the elderly [29]. A vaccine formulated with a formalin-inactivated virus (FI-RSV) and aluminum hydroxide was developed and the first clinical trial was conducted in the early 1960s. However, the vaccine failed to protect against RSV infection. Moreover, in a number of cases, enhanced pulmonary disease was observed in vaccinees upon subsequent natural infection by RSV [30,31]. This halted the development of an RSV vaccine for several decades. It is only within the past 10 years that the type of immune response induced by vaccination with FI-RSV has been analyzed and dissected in an animal model, leading to a hypothesis explaining the cause for failure of protection and the induction of enhanced disease by the vaccine [32]. In brief, the FI-RSV vaccine induced both low levels of neutralizing antibodies and an almost exclusively Th2-biased T-cell immune memory response (consistent with what is known about the activity of aluminum salts), whereas a Th1-type immune memory response is induced by natural RSV infection. The combined lack of adequate neutralizing antibody, which permitted infection to be established, and induction of Th2-type T-cell responses has been suggested as the basis for the aluminum-adjuvanted vaccine's failure in the 1960s [33].

In an attempt to develop a safe prophylactic vaccine against RSV disease capable of inducing a Th1-type response, the FI-RSV vaccine was adjuvanted with AS04 to favor TLR4 activation. Using the cotton rat model, it was demonstrated that immunization with FI-RSV/AS04 did not produce histological signs of enhanced disease upon further challenge with the virus, in contrast to the FI-RSV/aluminum hydroxide vaccine [34]. The same observation was made when AS04-adjuvanted recombinant F and G surface glycoproteins were tested [35]. Although such results are encouraging in the context of RSV vaccine development, more recent data tend to show that the mechanisms underlying RSV immunity might be more complex than foreseen and work is still ongoing to understand better the mechanisms by which MPL induces protective vaccination.

Epstein-Barr vaccine

EBV is prevalent in human populations worldwide and is responsible for infectious mononucleosis in adults and adolescents [36]. The symptoms of infection, recognizable as a general feeling of fatigue and malaise, may last for several months. In rare occasions, infectious mononucleosis may lead to more serious complications. Furthermore, a subset of host B cells remains latently infected in the individual. This reservoir of virus-infected cells accounts for long-term persistence of the infection, which may ultimately lead to different types of lymphomas, such as Kaposi's, which is common in Africa.

The most promising target protein for vaccination against EBV infection is gp350, a glycoprotein present at the surface of the viral capsid. The efficacy of such an approach was demonstrated in animal models [37]. In addition, a clinical trial involving a gp350-based vaccine was conducted in the early 1990s with encouraging results [38]. However, development of this vaccine appears to have been abandoned since then.

More recently, we have developed a gp350-based EBV vaccine adjuvanted with AS04. It is the first vaccine to demonstrate clinical efficacy in alleviating disease manifestations of infectious mononucleosis in a young adult population (FIGURE 9)

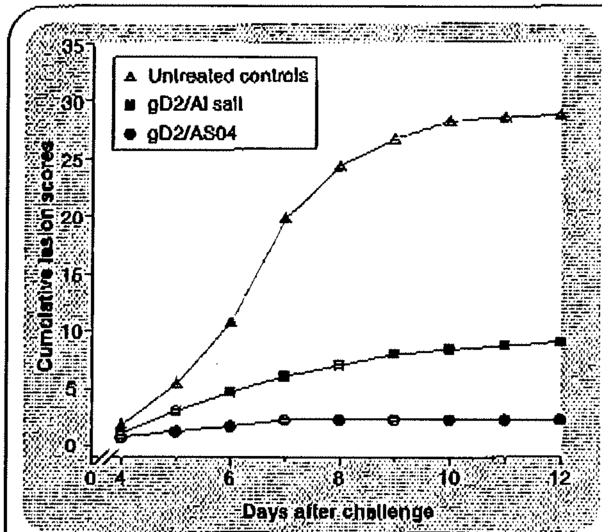

Figure 8. Assessment of vaccine efficacy against herpes simplex virus (HSV)-2 after *in vivo* virus challenge. Guinea pigs were immunized at days 0 and 28 with HSV type 2 glycoprotein D adjuvanted with aluminum salt or AS04. All guinea pigs were challenged intravaginally with HSV-2 strains at 29 days after the last immunization and were then monitored daily for clinical signs of acute disease. The severity of each lesion observed was scored on a 1–16 scale (0 for no lesions; 0.5–1 for vaginal lesions; and 2, 4, 8 and 16 for external skin lesions). The cumulative lesion scores (days 4–12) were calculated from the mean daily scores.
Based on data from [13].

[39]. However, further studies are needed to determine whether this novel EBV vaccine is able to control EBV latency in B-cell reservoir and to prevent the associated complications.

Safety

In the clinical trials listed previously, and over a period of more than 15 years, the safety of the AS04 formulation has been evaluated in humans. The AS04-based vaccines are generally well tolerated and have not raised any safety concerns. However, solicited local symptoms are usually reported. More particularly, and independent of the antigen combined with AS04, a greater proportion of vaccinees reporting soreness and swelling at the injection site is observed with AS04-adjuvanted vaccines compared with placebo or aluminum salt-only formulations; and the severity grade increases slightly [21,28,40–44]. This might, however, reflect a stronger immunological stimulation, with involvement of cell-mediated immunity that is induced by AS04 adjuvantation. Considering all studies, which now represent a clinical experience with more than 30,000 subjects and over 90,000 doses administered, these local reactogenicity symptoms are usually of short duration and overwhelmingly classified as mild-to-moderate. Very few reports of grade-3 pain for solicited symptoms were recorded and, when present, all were transient or resolved spontaneously. Laboratory safety monitoring has revealed no safety concerns. Furthermore, unsolicited adverse events reported were not qualitatively or quantitatively different from those in the control groups. Altogether, data generated to date demonstrate an acceptable tolerability and safety profile for AS04.

AS02-formulated vaccines

The AS02 Adjuvant System is the combination of an o/w emulsion with MPL and QS21. AS02 formulations have been developed with the objective of inducing high antibody titers, as well as to allow for a strong cell-mediated immune response, as characterized by the induction of high levels of IFN-γ, a marker of a CD4-type cellular response. The o/w emulsion was designed to elicit the optimal humoral and cellular responses when used alone or in combination with QS21 and/or MPL. QS21 is known to induce antigen-specific CTL responses in animal models [45,46]. MPL engages TLR4-dependent immune responses. Although initially developed for malaria, AS02 has been used in a number of other vaccine candidates against complex pathogens, when a strong T-cell response is needed to afford protection.

Malaria vaccine

Malaria, a major health problem in endemic areas, is caused by multistage protozoan parasites of the genus *Plasmodium*, with *Plasmodium falciparum* being responsible for the most severe disease and accounting for most of the deaths recorded. Malaria kills 1–2 million people annually, mostly children under 5 years of age in sub-Saharan Africa. The emergence of drug-resistant parasites and of insecticide-resistant mosquitoes has highlighted the urgency to develop an effective vaccine.

The vaccine developed by GSK in collaboration with the Walter Reed Army Institute of Research (WRAIR, DC, USA) was designed to target the pre-erythrocytic stage of the parasite, with a dual goal in mind: generating an antibody response able to neutralize sporozoites and prevent them from invading hepatocytes, and eliciting a cell-mediated immune response in order to interfere with the intrahepatic stage of the parasite by killing the infected hepatocytes or impairing the development of intrahepatic parasites. AS02 was selected for our candidate malaria vaccine to meet these immunological requirements. The antigen is derived from the circumsporozoite protein (CSP) that is found at the surface of the sporozoite stage, as well as during the early stages of parasite development in the hepatocytes. It consists of a recombinant protein including portions of the CSP fused to HBV surface antigen (HBsAg), together with unfused HBsAg, all expressed in genetically engineered yeast cells. When purified, this antigen mixture spontaneously assembles into multimeric particles, named RTS,S [47].

During preclinical studies in mice, a synergistic effect was observed between QS21 and MPL as illustrated by the induction of CSP-specific CTLs. It appeared that, although AS04 proved to be efficient in other contexts, AS02 was the best candidate for a malaria vaccine. This combination of o/w emulsion, MPL and QS21 was selected on the basis of results from monkey studies evaluating various Adjuvant Systems and on the basis of the antibody level induced and the cell-mediated immunity generated (reviewed in [48]).

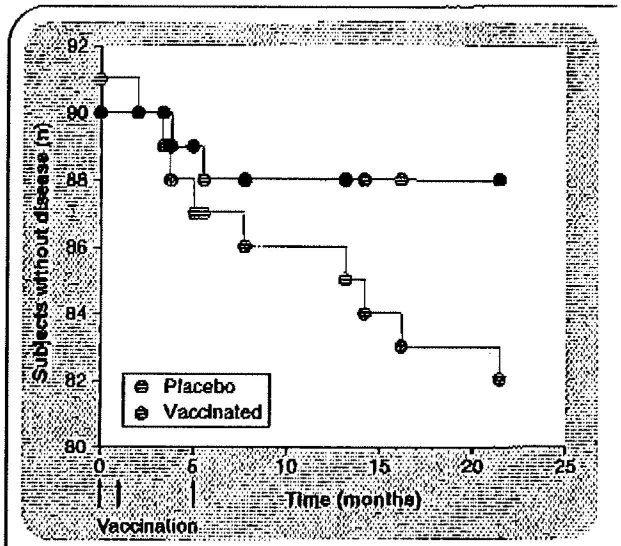

Figure 9. Timing of occurrence of infectious mononucleosis cases in vaccine and placebo recipients. Epstein–Barr virus-negative human subjects were vaccinated with AS04-adjuvanted gp350 formulation or with placebo. After completion of the vaccination regimen, no additional case of infectious mononucleosis was reported in the vaccinated group, while cases still occurred in the placebo group ($p = 0.0468$).
Based on data from [39].

In an initial Phase I/IIa clinical trial conducted at the WRAIR, the superiority of the candidate vaccine containing AS02 was demonstrated over two alternative candidate vaccines containing other Adjuvant Systems, protecting six out of seven volunteers (86%) against infection by *P. falciparum* parasites delivered through the bites of infectious mosquitoes in a laboratory-based challenge model [47]. This was the first report of a malaria vaccine capable of inducing significant protection against infection in humans. Several subsequent clinical trials were conducted to ascertain the safety of the formulation in adult volunteers living in malaria endemic regions [49] and to evaluate various vaccination schedules [50]. Following these encouraging results, a Phase IIb double-blind, randomized, controlled study in semi-immune adults was conducted in The Gambia and provided the first demonstration of the efficacy of RTS,S/AS02 vaccination against natural challenge in a field setting, showing a vaccine efficacy against infection of 71% during the first 9 weeks of follow-up [51]. Clinical trials have continued, targeting other exposed populations. In a proof-of-concept trial including 2022 children 1–4 years of age in Mozambican rural endemic areas, the efficacy of three doses of RTS,S/AS02 at preventing a first malaria attack was approximately 29.9% and the incidence of severe disease was decreased by 57.7% over a 6-month follow-up [52]. After 18 months, vaccine efficacy was 35.3% against cases of uncomplicated malaria and still 48.6% against severe disease [53].

RTS,S/AS02 is the only clinically evaluated vaccine candidate to date able to elicit a protective immune response against *P. falciparum* infection and prevent disease. The success of the vaccine candidate is due to the rational design and combination of antigen and Adjuvant System. It has been established that, in addition to the high specific antibody titers observed in all studies, the vaccine induced CSP-specific $CD4^+$ T cells in human vaccinees [54–58]. CSP-specific $CD8^+$ cells were also demonstrated in one study [57]. Although the antibody titers were shown to wane over time, the elicitation of different population of T cells by the vaccine may contribute to protective efficacy and may help in making the immune response recallable upon revaccination or subsequent infection. These clinical results largely confirm preclinical data and ***a posteriori*** validate the approach taken to rationally design this vaccine.

Alternative malaria antigens have been evaluated alone or in combination with AS02. One of them, MSP1, has been tested in monkeys. AS02 was superior to alum and to other adjuvants in the induction of specific antibody. The immune response elicited by MSP1/AS02 was described as balanced Th1/Th2, with antigen-specific IFN-γ-producing cells detectable for up to 24 weeks following vaccination [59–61].

Several additional Phase II trials are now ongoing in various African countries to evaluate a number of vaccine parameters and its performance in younger age group. A large multicentric Phase III clinical trial with RTS,S is planned to start in late 2008 in eight to ten sites across sub-Saharan Africa.

Tuberculosis vaccine

Tuberculosis, despite widespread use of bacillus Calmette–Guérin (BCG) vaccination, is still responsible for 1.6 million deaths each year worldwide. BCG is the only currently accepted vaccine for tuberculosis, with more than 3 billion people vaccinated worldwide. However, its efficacy remains somewhat controversial. This is largely due to the fact that while BCG effectively protects infants from tuberculosis, immunity declines with age and fails to protect adults against pulmonary tuberculosis, the primary source of dissemination [62–64].

With the advent of molecular biology, new vaccine strategies can be envisioned using better-defined recombinant proteins. The association of such recombinant proteins with Adjuvant Systems opens new perspectives for improved vaccines for tuberculosis. One of the tuberculosis-derived recombinant vaccine antigens, Mtb72f, is a fusion protein based upon the Mtb32A and Mtb39A antigens of *Mycobacterium tuberculosis*. Mtb72F adjuvanted with AS02 has been shown to induce a moderate IFN-γ and a weak CTL response to the antigen in mice. This response was sufficient to protect the animals from a challenge with *M. tuberculosis* [65]. Moreover, immunization of guinea pigs with Mtb72f formulated in AS02 conferred a prolonged (>1 year) protective immune response against an aerosol challenge with virulent *M. tuberculosis*; this protection was similar to BCG immunization [65]. In a rabbit model of tuberculous meningitis, Mtb72f/AS02 vaccination induced protection similar to that induced by BCG against CNS bacterial challenge, as seen by the clearance of bacilli from the cerebrospinal fluid, the reduced leukocytosis and less pathology of the brain and the lungs [66]. In an open-label Phase I trial conducted in humans with Mtb72f

formulated in AS02, anti-Mtb72f antibodies were detected at 2 weeks after the second dose and higher levels were induced after the third dose. Cell-mediated immune responses measured by intracellular cytokine staining after a short-term *in vitro* restimulation with a pool of peptides covering the sequence of Mtb72f revealed antigen-specific CD4+ T cells, but no specific CD8+ T cells [67]. This demonstrated again the ability of AS02 to impact positively on both the humoral and cell-mediated immune responses.

Other *M. tuberculosis* antigens have been evaluated with AS02, including Mtb41 and Ag85B–ESAT-6. MTB41 was shown to induce a strong specific CD4+ T-cell response in mice, but no MHC class I-restricted CTL activity, and to confer protection in guinea pigs against a challenge with a virulent *M. tuberculosis* [68]. The Ag85B–ESAT-6 fusion protein was evaluated in cynomolgus monkeys, leading to a reduction in bacterial number and/or lung pathology in the vaccinated animals after challenge with *M. tuberculosis* [69].

These results are encouraging and further work is ongoing to develop an improved tuberculosis vaccine. Alternative Adjuvant Systems may be evaluated as well as different prime–boost vaccination strategies.

Hepatitis B vaccine

In patients who have undergone liver transplant due to HBV infection, there is a requirement for a lifelong hepatitis B hyperimmunoglobulin (HBIG) treatment that, to date, cannot be substituted. AS02, with its ability to induce high and persisting antibody titers as seen in the context of the malaria vaccine, has been evaluated in candidate vaccines for this target population. A total of 80% of subjects responded positively to the vaccination with a high antibody response (GMT of 7293 mIU/ml; range 721–45,811 mIU/ml), allowing them to suspend HBIG treatment [70]. In another study, by using recombinant SL*, a protein combining the small HBV envelope protein and parts of the large protein, SL*/AS02 was capable of inducing not only strong humoral and Th-cell responses, but also CTL responses in all HLA-A2-positive and -negative patients (FIGURE 10) [71].

These studies demonstrated the potential of the AS02 Adjuvant System, but also emphasize again the need to combine it with the right antigen in order to elicit the proper immune response, be it humoral or cell-mediated through CD4+ or CD8+ T cells.

HIV vaccine

Many different approaches have been evaluated to develop a vaccine against HIV/AIDS. These mostly involve use of recombinant vectors or recombinant proteins, all based on the most relevant conserved antigens identified on the HIV to elicit cell-mediated immune responses or envelope protein-derived antigens to generate neutralizing antibodies. We initially evaluated a vaccine based on the combination of AS02 and the envelope protein gp120. AS02 adjuvantation of gp120 was shown to induce protection in primed and naive monkeys when challenged with the vaccine-homologous HIV strain [72]. Such a recombinant monomeric approach, however, proved to be too strain specific. Following these results, a different strategy was developed, based on the use of an AS02-adjuvanted multicomponent formulation, comprising a recombinant derivative

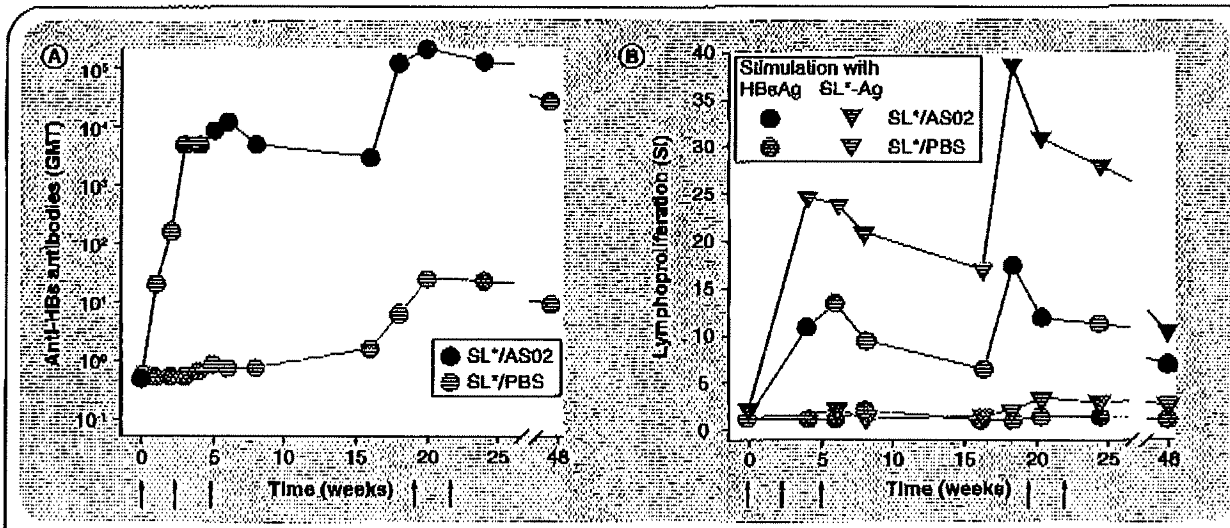

Figure 10. Evaluation of the immune response after vaccination with AS02–adjuvanted hepatitis B vaccine. Human subjects were vaccinated with SL*, a recombinant hepatitis B protein, adjuvanted with AS02 or in phosphate buffered saline (PBS). Blood samples were taken at different time points during and after the course of the immunization regimen. Specific anti-HBsAg levels were measured by ELISA (A), and the lymphoproliferative response upon stimulation with SL* or HBsAg was evaluated by thymidine incorporation assay (expressed as SI) (B). The arrows indicate the times of injections. The anti-HBsAg levels were significantly lower in the PBS group than in the AS02 adjuvant group for all time points ($p < 0.001$). For lymphoproliferation, at all time points the response was statistically better in the group vaccinated with SL*/AS02 than that with SL*/PBS ($p < 0.001$).
GMT: Geometric mean titer; SI: Stimulation index.
Based on data from [71].

of gp120 and regulatory viral proteins, such as Tat and Nef. This vaccine was first studied in monkeys, where it demonstrated efficacy in reducing virus load and protecting against decline in CD4+ T cells [73]. Furthermore, this approach has been evaluated in humans, where high levels of specific antibodies and, most importantly, very strong Th-cell responses have been detected [74]. The antibodies generated, however, were not able to neutralize primary isolates of the virus, but only laboratory-modified strains. New vaccine strategies, built on the results observed with AS02, are being evaluated.

Cancer immunotherapy

As mentioned previously, persistent infection with some viruses, such as HPV, EBV or HBV, can lead to the development of tumors (cervical cancer, lymphomas or hepatocarcinomas, respectively) that express virus-derived antigens. In such cases, prophylactic vaccination, by combating the virus infection, is aimed at protecting the individual against the development of the corresponding tumors. However, for cancers that are not induced by viruses, a therapeutic strategy is the preferred option.

One of the reasons for the progression of tumors is that, despite the expression of tumor-specific antigens, they lack immunogenicity and escape immune surveillance. In the therapeutic approach that GSK is developing, it is speculated that the use of tumor-specific antigens together with potent Adjuvant Systems could stimulate natural immune responses, more particularly cytolytic T-cell activity. In fact, with this approach the immune system learns to recognize and eliminate the cancer cells specifically. In that context, another Adjuvant System, part of the AS02 family, has been tested.

Several cancer immunotherapy studies with AS02 and the antigen MAGE-A3, a highly tumor-specific antigen found in several tumor-types, such as melanoma and non-small-cell lung cancer, have been conducted. Patients were selected with MAGE-A3-positive advanced tumors (mostly melanomas) and received four immunotherapeutic injections (up to six for the best responders) at different time intervals [75,76]. The results show that 96% of the patients displayed a significant anti-MAGE-A3 antibody response. In addition, among the evaluable patients, 30 and 14% showed increased IFN-γ and IL-5 production, respectively, upon *in vitro* MAGE-A3 stimulation. In another study, the immune responses induced by the same MAGE-A3/AS02 vaccine were evaluated in lung cancer patients [77]. The injections elicited strong antigen-specific humoral and CD4+ T-cell response, associated with CD8+ response in some cases. Immunotherapy with MAGE-A3 antigen in AS02 is able to induce MAGE-A3-specific antibody and T-cell responses, hence to stimulate and enhance the immune system. It thereby potentially impacts on tumor regression. These studies have led to the development of the GSK concept of antigen-specific cancer immunotherapy, referred to as ASCI.

Safety

In all studies performed, the AS02-adjuvanted vaccines were well tolerated, with the most frequent adverse event being mild-to-moderate adjuvant-related swelling and pain at the injection sites. Some of the studies, however, were more specifically conducted to evaluate the safety and reactogenicity of this Adjuvant System, mainly in the context of the malaria vaccine. The vast majority of local and constitutional adverse events (pain and swelling at the injection site, as well as headache, fatigue and gastrointestinal symptoms) resolved within 24–48 h with no sequelae [49–51,60,67,78,79]. No significant hematological or biochemical abnormalities that could have been induced by vaccination were reported. The frequency and severity of the reported adverse events do not, in general, increase with repeated vaccination. However, increased reactogenicity has been reported occasionally and appears to be vaccine specific. Interestingly, the rates of local and general adverse events were lower in children than in adults. As was seen in adults, the most frequent adverse events in children were swelling and pain at the injection site. Rates for grade 3 events were rather low and were transient (<48 h) [52,61,80].

AS01-formulated vaccines

Owing to the weak CD8+ response observed in various clinical trials so far, a specific attempt has been made to improve the cell-mediated immunity induced by the Adjuvant Systems through an alternative formulation, AS01, composed of liposomes, MPL and QS21.

Malaria vaccine

To date, the immune response induced by the current AS02-adjuvanted candidate vaccine allows for an unprecedented protection against *P. falciparum* infection and malaria clinical disease, bringing hope for an additional instrument in our armory of prevention weapons. To further increase the immune response, and in particular the immunity required to eliminate infected hepatocytes, the AS01 Adjuvant System has been evaluated in the context of RTS,S as an alternative approach to RTS,S/AS02. In an adjuvant comparative study in rhesus macaques, AS01 elicited higher RTS,S-specific antibody titers than did AS02. The number of antigen-specific IFN-γ-producing cells was also higher with RTS,S/AS01 than with any other formulation, indicative of a better induction of T-cell responses [81]. In a heterologous prime–boost vaccination strategy [82], monkeys were first immunized with nonreplicating adenovirus encoding CSP, then boosted with RTS,S/AS01. High antibody titers were induced, as well as long-lasting (up to 6 months) high levels of Th1-type IFN-γ-producing cells (FIGURE 11).

Similar observations were made with another malaria antigen, MSP1(42), a specific merozoite stage protein [59]. In this comparative study, AS02 and AS01 formulations elicited similar antibody responses against MSP1(42), which were significantly higher compared with other adjuvant groups. By ELISPOT assays, it was demonstrated that, whereas AS02 induces balanced Th1/Th2 responses, AS01 clearly favors Th1 responses, as shown by the higher levels of IFN-γ-producing cells and lower levels of IL-5-producing cells. In another experiment, LSA-1, a liver stage-specific malaria antigen, was studied in association with AS02 or AS01 in different mouse strains [83]. In the responder murine strains, a stronger Th1 response was induced by AS01 compared with AS02, whereas the latter was

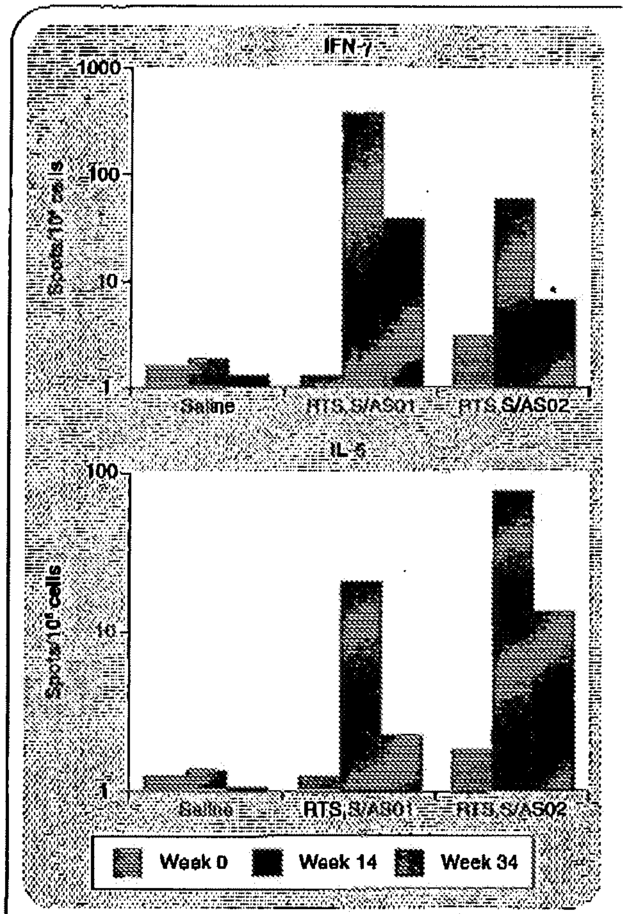

Figure 11. T-cell responses induced by two different formulations of the malaria vaccine antigen RTS,S. Monkeys were immunized against malaria by three injections of RTS,S adjuvanted with either AS02 or AS01, or in saline. The number of antigen-specific IFN-γ- and IL-5-producing cells were estimated by ELISPOT at 2 and 22 weeks after the third injection (weeks 14 and 34, respectively). Significant differences ($p < 0.015$) between the cytokine levels induced by the AS01 and the AS02 formulations are indicated by an asterisk.
Based on data from [81].

responsible for a higher specific antibody titer. CD4⁺, but not CD8⁺, cells were the main producers of IFN-γ in the mice in response to AS01 adjuvantation.

A comparative challenge study in humans has demonstrated the superiority of AS01-adjuvanted vaccine versus the AS02-adjuvanted one in terms of antibody titers and cell-mediated immunity. Furthermore, a trend toward better protection with the AS01 formulation has been observed. Based on these results (KESTER K, PERS. COMM.), the AS01 formulation of RTS,S is now being evaluated in field studies in adult and pediatric populations.

Tuberculosis vaccine

To follow-up on the results obtained with AS01 for the induction of cell-mediated immunity to malaria, the same Adjuvant System was evaluated in the context of tuberculosis. In a rabbit model of tuberculous meningitis, a vaccine containing recombinant polyprotein Mtb72f and AS01 was compared with a formulation of the same antigen in AS02, BCG alone, or a combination of BCG-prime/Mtb72f-boost regimen [66]. AS01 was as efficient as AS02 in protecting the animals from CNS mycobacterial challenge. Interestingly, AS01-adjuvanted Mtb72f vaccine could boost the immunity against tuberculosis in BCG-primed rabbits without exacerbation of leukocytosis and weight loss.

In another study in mice, the added value of AS01 in the context of the Mtb72f vaccine was demonstrated, as compared with the DNA approach, for the induction of CD4⁺ and CD8⁺ responses [65]. Adjuvantation with AS02 induced weaker T-cell responses. However, all three vaccine approaches resulted in the protection of the animals against aerosol challenge with a virulent strain of *M. tuberculosis*, which questions the need for cytolytic T-cell responses in this model. Differences between the immunogenicity of protein formulated in AS02 versus AS01 were also observed in the cynomolgus monkey model. In animals immunized with Mtb72f formulated in AS01, a higher number of antigen-specific IFN-γ ELISPOTS was observed as well as an increased lymphoproliferation. Similar effects of adjuvant were seen when measuring IFN-γ secretion by peripheral blood mononuclear cells from these monkeys; AS01 induced higher levels of antigen-specific cytokine than AS02 [84]. Nonetheless, the global level of the immune responses observed with AS01 justifies further evaluation of this vaccine candidate in human trials.

Safety

To date, AS01 has been administered to a limited number of clinical trial volunteers and has been generally well tolerated thus far. Further evaluation in clinical conditions will be required to ascertain the reactogenicity profile of this Adjuvant System. Preclinical safety evaluations, as well as a monkey study, have demonstrated a safety pattern similar to that observed to date with other Adjuvant Systems [59].

Manufacture of Adjuvant Systems

In addition to the demonstration of safety, immunogenicity and protection in preclinical (where a model exists) and clinical studies, any Adjuvant System will need to demonstrate feasibility of large-scale manufacturing. This includes definition, selection, supply and characterization of the raw materials, as well as the development of production processes that will deliver a stable and consistent product. In order to reach the final product, manufacturing processes, analytical and immunological tools, and quality control testing need to be conducted successfully. Preclinical toxicology studies need to be designed on a case-by-case basis and performed with the adjuvant alone and in combination with the antigen of interest, according to the existing guidelines. All these steps are critical for the delivery of reliable Adjuvant System to be produced at large scale, will take several years to be completed and will require the deployment of resources in many areas of vaccinology and process development (FIGURE 12). Only through this strict and controlled process will the quality of future adjuvanted vaccines be guaranteed.

Conclusion

Over the past decades, the development of adjuvants has gradually moved from an exclusively empirical to an increasingly rational design thanks to the progress made in understanding the immune system as well as to the improvements made on analytical, chemical and immunological tools.

Of the many adjuvants tested preclinically in vaccine formulations by the vaccine research community, only few have been evaluated in humans and even fewer yet included in registered or marketed vaccines. It is possible today to optimally combine the appropriate Adjuvant System with the right antigen for the design of innovative vaccines that will provide a tailored immune response adapted to the pathogen and to the target population. Novel Adjuvant System technology is fundamental to the development of new or more effective vaccines for very challenging diseases or for subjects with immunologically challenging conditions where classical approaches have proven less effective or have failed.

Adjuvant Systems have been developed that can induce a stronger humoral and cell-mediated immune response correlating with better protection. In various vaccine applications, the AS04 family of Adjuvant Systems enables stronger and sustained immune response, which correlates with an improved protection (e.g., against HBV in a high-risk population) or may explain the 5.5-year sustained protection against precancerous lesions associated with HPV-16 and -18 types. The AS02 family of Adjuvant Systems has demonstrated its ability to help vaccines induce a stronger and protective immune response in areas of huge unmet medical needs to a point where an efficacious and safe vaccine against malaria can now be contemplated. Because other vaccines may need additional potentiality than afforded by AS02 formulations, the AS01 family of Adjuvant Systems has been developed. It demonstrates its ability to expand the cell-mediated immune response, leading to improved vaccines as well as new areas of vaccinology.

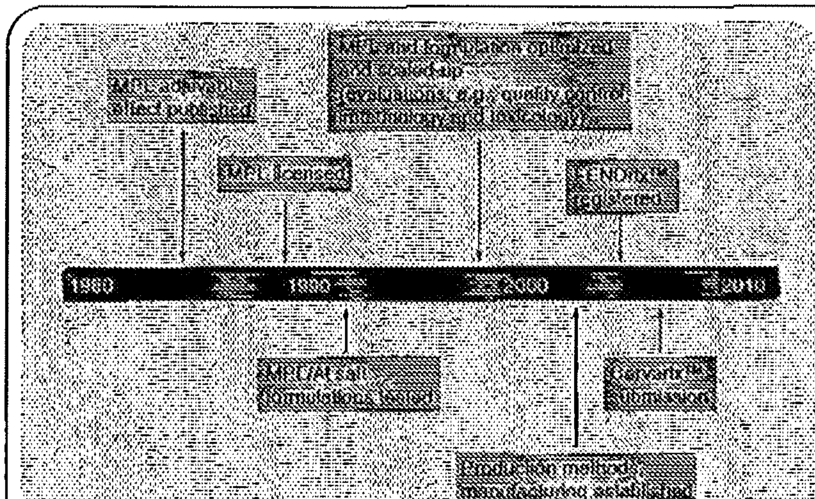

Figure 12. Timeline of significant events over the development of AS04.
MPL: 3-*O*-desacyl-4'-monophosphoryl lipid A.

Expert commentary & five-year view

During the past century, the evolution of vaccinology has moved from empirism and intuition to rational design. This has been made possible by a better understanding of the host–pathogen interactions and the underlying immune mechanisms. Those advances have allowed new approaches that combine target antigens produced by recombinant DNA technology with Adjuvant Systems designed to trigger the appropriate immune response. On a purely immunological basis, however, one of the remaining challenges for the Adjuvant Systems in the near future is the efficient induction of the $CD8^+$ arm of immunity. Nevertheless, new vaccines able to face huge unmet medical needs (e.g., malaria and cancer, or vaccines better adapted to the target population, such as immunocompromised patients or the elderly) now come into reach thanks to the Adjuvant Systems. This raises expectations for comparable clinical successes in other diseases, such as HIV, TB or RSV, where vaccines are still in development. In addition, Adjuvant Systems have been shown to reduce the dosage required to stimulate the appropriate immune response to create effective immunity against a specific disease. This enables dose sparing and dose recovery where antigen supply is limited, as is currently the case for pandemic influenza vaccines.

Adjuvant Systems also bring hope for new areas of vaccinology, such as therapeutic vaccines (HBV and HCV) and chronic disorders (allergy, autoimmunity or addictions). Research remains essential to unravel the molecular mechanisms underlying the dysregulation present in these affections. This knowledge will be useful for the determination of the optimal combination of Adjuvant System(s) and antigen(s).

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Key issues

- Purified recombinant or subunit antigens, as used in modern vaccines, are innocuous but also often not sufficiently immunogenic, which imposes the use of adjuvants in order to stimulate the immune response of the vaccinees.
- Current improved knowledge of immunity, particularly of the interdependence of the innate and adaptive immune systems, allows us to understand how a vaccine should work for a specific disease.
- Adjuvant Systems are unique combinations of classical adjuvants and immunomodulators specifically designed to activate desired arms of the immune system, in order to be adapted to the pathogens and to the targeted populations.
- The concept of Adjuvant System has allowed some major breakthroughs in vaccinology, such as in the fight against human papillomavirus, with an AS04-adjuvanted vaccine, and in the fight against malaria, with an AS02-adjuvanted vaccine.
- The Adjuvant System family is growing, giving birth to new improved vaccines and designating new potential vaccine targets.

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